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(54) Title: METHODS FOR IDENTIFYING PROTEINS WITH STARCH PHOSPHORYLATING ENZYMATIC ACTIVITY

(57) Abstract: The present invention relates to a method for identifying proteins involved in the phosphorylation of starch and nucleic acids which code for such proteins. The present invention further relates to plant cells and plants which exhibit an altered activity of a protein which can be identified using the method according to the invention. Plant cells and plants of this type synthesise a modified starch. The present invention therefore also relates to the starch synthesised by the plant cells and plants according to the invention as well as to methods for the manufacture of this starch and to the manufacture of starch derivatives of this modified starch.

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Methods for identifying proteins with starch phosphorylating 5 enzymatic activity

Description

The present invention relates to a method for identifying proteins involved in the
10 phosphorylation of starch, and nucleic acids which code for such proteins. The
invention further relates to plant cells and plants which exhibit an elevated activity of
a protein identifiable using the method according to the invention. Plant cells and
plants of this type synthesise a modified starch. The present invention therefore also
relates to the starch synthesised by the plant cells and plants according to the
15 invention as well as to methods for the manufacture of this starch and to the
manufacture of starch derivatives of this modified starch.

With regard to the increasing importance currently attributed to plant constituents as
renewable raw material sources, one of the tasks of biotechnological research is to
20 endeavour to adapt these plant raw materials to suit the requirements of the
processing industry. Furthermore, in order to enable regenerating raw materials to be
used in as many areas of application as possible, it is necessary to achieve a large
variety of materials.

25 Polysaccharide starch is made up of chemically uniform base components, glucose
molecules, but constitutes a complex mixture of different molecule forms, which
exhibit differences with regard to the degree of polymerisation and branching, and
therefore differ strongly from one another in their physical-chemical characteristics.

Discrimination is made between amylose starch, an essentially unbranched polymer made from alpha-1,4-glycosidically linked glucose units, and amylopectin starch, a branched polymer, in which the branches come about by the occurrence of additional alpha-1,6-glycosidic links. A further essential difference between amylose and amylopectin lies in the molecular weight. While amylose, depending on the origin of the starch, has a molecular weight of 5×10^5 - 10^6 Da, that of the amylopectin lies between 10^7 and 10^8 Da. The two macromolecules can be differentiated by their molecular weight and their different physical-chemical characteristics, which can most easily be made visible by their different iodine bonding characteristics.

10

Amylose has long been regarded as a linear polymer, consisting of alpha-1,4-glycosidically linked alpha-D-glucose monomers. In more recent studies, however, the presence of alpha-1,6-glycosidic branching points (ca. 0.1%) has been shown (Hizukuri and Takagi, Carbohydr. Res. 134, (1984), 1-10; Takeda et al., Carbohydr. Res. 132, (1984), 83-92).

The functional properties such as, for example, the solubility, retrogradation behaviour, water binding capacity, film-forming properties, viscosity, gelatinisation properties, freeze-thaw stability, acid stability, gel strength and the starch granule size of starches are influenced among other things by the amylose/amylopectin ratio, molecular weight, side-chain distribution pattern, ion content, lipid and protein content, average starch granule size, starch granule morphology etc. The functional properties of starch are also influenced by the phosphate content, a non-carbon component of starch. Here, differentiation is made between phosphate, which is bound covalently in the form of monoesters to the glucose molecules of the starch (described in the following as starch phosphate), and phosphate in the form of phospholipids associated with the starch. In addition to the phosphate content, the influence on the functional properties of the starch is in this case also dependent on the form (starch phosphate or phospholipid) in which the phosphate occurs in the starch (Jane et al., 1996, Cereal Foods World 41 (11), 827-832).

The starch phosphate content varies according to the species of plant. Therefore, certain maize mutants, for example, synthesise a starch with increased starch phosphate content (waxy maize 0.002% and high-amylose maize 0.013%), while conventional types of maize only have traces of starch phosphate. Likewise small quantities of starch phosphate are found in wheat (0.001%) whereas no starch phosphate could be detected in oats and sorghum. In rice mutants likewise, less starch phosphate was found (waxy rice 0.003%) than in conventional species of rice (0.013%). Significant quantities of starch phosphate were detected in tuber- or root-storing starch synthesising plants such as, for example, tapioca (0.008%), sweet potato (0.011%), arrowroot (0.021%) or potato (0.89%). The percentage values for the starch phosphate content quoted above refer to the dry weight of starch in each case, and have been determined by Jane et al. (1996, Cereal Foods World 41 (11), 827-832).

Starch phosphate can be present in the form of monoesters at the C-2, C-3 or C-6 position of the polymerised glucose monomers (Takeda and Hizukuri, 1971, Starch/Stärke 23, 267-272). The phosphate distribution of the starch phosphate in starch synthesised by plants is generally distinguished by the fact that about 30% to 40% of the phosphate residues are covalently bound in the C-3 position and about 60% to 70% of the phosphate residue is covalently bound in the C-6 position of the glucose molecule (Blennow et al., 2000, Int. J. of Biological Macromolecules 27, 211-218). Blennow et al. (2000, Carbohydrate Polymers 41, 163-174) have determined a starch phosphate content which is bound in the C-6 position of the glucose molecule for various starches, such as for example, potato starch (between 7.8 and 33.5 nMol per mg of starch, depending on the type), starch from various *Curcuma* species (between 1.8 and 63 nMol per mg), tapioca starch (2.5 nMol per mg of starch), rice starch (1.0 nMol per mg of starch), mung bean starch (3.5 nMol per mg of starch) and sorghum starch (0.9 nMol per mg of starch). These authors have been unable to show any starch phosphate bound at the C-6 position in barley starch and starches from different waxy mutants of maize. Up to now, it has not been possible to establish a connection between the genotype of a plant and the starch phosphate content (Jane et al., 1996, Cereal Foods World 41 (11), 827-832). Thus, at the

present time it is not possible to influence the content of starch phosphate in plants by means of breeding.

In transgenic plants the quantity of starch phosphate in storage starches can be varied. Thus, storage starch from potato plants which exhibit a reduced activity of soluble starch synthase III (Abel et al., 1996, *The Plant Journal* 10(6), 9891-991), branching enzyme I (BEI) (Safford et al., 1998, *Carbohydrate Polymers* 35, 155-168), branching enzyme II (BEII) (Jobling et al., 1999, *The Plant Journal* 18, 163-171), BEI and BEII (Schwall et al., 2000, *Nature Biotechnology* 18, 551- 554), a disproportionation enzyme (WO 96 27673) or a disproportionation enzyme and a BEI (WO 95 07355), show an elevated content of starch phosphate compared with starch from corresponding wild type plants. However, the alteration in the starch phosphate content in these plants is not due to the proteins whose activity is reduced in these plants, being directly involved in the introduction of phosphate residues into the starch. The increase in the content of starch phosphate in the transgenic plants concerned is thus not a primary but a secondary effect which is brought about by reduction of the corresponding proteins. The reason for the increase in the content of starch phosphate as a result of modification of said protein activities is as yet still unexplained. Thus, it is not possible to specifically modify the content of starch phosphate by modifying protein activities which only influence the starch phosphate content by a secondary effect. Furthermore, modifying the activities of proteins which as a secondary effect have an influence on the content of starch phosphate in plants, at the same time also brings about further modifications in the starch, such as, for example: changes in the amylose/amylopectin ratio and/or the length of the side chains of the amylopectin which constitutes the primary effect of the changes in such protein activities.

Previously, only one protein has been described, which mediates the introduction of covalent bonds of phosphate residues to the glucose molecules of starch. This protein, frequently designated as R1 in the scientific literature, is bound to the starch granules of the storage starch in potato tubers (Lorberth et al., 1998, *Nature*

Biotechnology 16, 473-477) and has the enzymatic activity of an alpha-glucan water
dikinase (E.C. 02.07.09.4). In the reaction catalysed by R1 the educts alpha-1,4-
glucan (starch), adenosinetriphosphate (ATP) and water are converted to the
products glucan phosphate (phosphorylated starch), monophosphate and adenosine
5 monophosphate. In this case, the gamma phosphate residue of the ATP is
transferred to water and the beta phosphate residue of the ATP is transferred to the
glucan (starch). R1 transfers *in vitro* the beta phosphate residue of the ATP to the C-
6 and the C-3 position of the glucose molecules of alpha-1,4-glucans. The ratio of C-
6 phosphate to C-3 phosphate which is obtained in the *in vitro* reaction corresponds
10 to the ratio which is present in starch isolated from plants (Ritte et al., 2002, PNAS
99, 7166-7171). As about 70% of the starch phosphate present in potato starch is
bound to the glucose monomers of the starch in the C-6 position and about 30% in
the C-3 position, this means that R1 preferably phosphorylates the C-6 position of the
glucose molecules. Furthermore, it has been shown by using amylopectin from
15 maize that, amongst other things, R1 can phosphorylate alpha-1,4-glucans which do
not yet contain covalently bound phosphate (Ritte et al., 2002, PNAS 99, 7166-7171),
i.e., R1 is able to introduce phosphate *de novo* into alpha-1,4-glucans.

The amino acid sequence of R1 contains a domain which exhibits a high degree of
20 homology to known pyruvate phosphate dikinases (PPDK domains) and known
pyruvate water dikinases (PPS domains) and contains a histidine residue conserved
in PPDK and PPS domains. During the transfer of phosphate residues of the ATP to
alpha-1,4-glucans (starch) a phosphorylated R1 protein is formed as intermediate
product, with a phosphate residue being present, covalently bound to the histidine
25 residue conserved in the PPDK or the PPS domain (Mikkelsen et al., 2004,
Biochemical Journal 377, 525-532).

Nucleic acid sequences and amino acid sequences corresponding to these, coding
for an R1 protein, are described from various species, such as for example, potato
30 (WO 97 11188, GenBank Acc.: AY027522, Y09533), wheat (WO 00 77229, US
6,462,256, GenBank Acc.: AAN93923, GenBank Acc.: AR236165), rice (GenBank

Acc.: AAR61445, GenBank Acc.: AR400814), maize (GenBank Acc.: AAR61444, GenBank Acc.: AR400813), soya bean (GenBank Acc.: AAR61446, GenBank Acc.: AR400815), citrus (GenBank Acc.: AY094062) and *Arabidopsis* (GenBank Acc.: AF312027).

5

Wheat plants which exhibit an elevated activity of an R1 protein as a result of overexpression of an R1 gene from potato are described in WO 02 34923. Compared with the corresponding wild type plants, in which no starch phosphate could be detected, these plants synthesise a starch having significant quantities of starch
10 phosphate in the C-6 position of the glucose molecules.

Further proteins which catalyse a reaction which introduces covalently bound phosphate groups into the starch have not so far been described. Enzymes, which preferably introduce phosphate groups in the C-3 position and/or the C-2 position of
15 the glucose molecules of starch, are also not known. Thus, apart from increasing the content of starch phosphate in plants, there are no available ways for specifically influencing the phosphorylation of starch in plants, modifying the phosphate distribution within the starch synthesised by plants and/or further increasing the content of starch phosphate.

20

It is thus the object of the present invention to provide methods and means for producing plants synthesising a modified starch having elevated phosphate content and/or modified phosphate distribution. as well as to provide plant cells and/or plants which synthesise such a modified starch.

25

This problem is solved by the embodiments described in the claims.

Thus, the present invention relates to a method for identifying a protein which has an elevated binding activity towards phosphorylated alpha-1,4 glucans, compared to non-phosphorylated alpha-1,4 glucans, wherein

- a) protein extracts in preparations separated from one another are incubated with
 - 5 i phosphorylated alpha-1,4 glucans and
 - ii non-phosphorylated alpha-1,4 glucans,
- b) proteins specifically bound to the
 - i phosphorylated alpha-1,4 glucans from step a) i and
 - ii proteins specifically bound to the non-phosphorylated alpha-1,4 glucans
 - 10 from step a) ii

are dissolved in preparations separate from one another and
- c) proteins are identified which exhibit an elevated binding activity towards phosphorylated alpha-1,4 glucans used in step b) i, compared to non-phosphorylated alpha-1,4 glucans used in step b) ii.

15

In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, the alpha-1,4 glucan to which a higher binding activity exists is a starch, preferably a granular starch.

20

A further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, relates to a method for identifying a protein which has a molecular weight derived from the amino acid

25 sequence of 120 kDa to 145 kDa, preferably 120 kDa to 140 kDa, particularly preferably 125 kDa to 140 kDa, especially preferably 130 kDa to 135 kDa.

In a further embodiment, the method according to the invention relates to a method for identifying a protein which exhibits an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, wherein the binding activity to P-alpha-1,4-glucans is increased at least three times, preferably at least
5 four times, particularly preferably at least five times and especially preferably at least six times compared to the binding activity to non-phosphorylated alpha-1,4-glucans.

The quantity of proteins which bind to P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans can, for example, be determined by immunological methods such
10 as Western Blot Analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio ImmunoAssay).

Methods for manufacturing antibodies, which react specifically with a certain protein, i.e. which bind specifically to said protein, are known to the person skilled in the art
15 (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad, Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). The manufacture of such antibodies is offered by some companies (e.g. Eurogentec, Belgium) as a contract service. One possible way for manufacturing antibodies which react specifically with a protein according to the invention is described below (see Example 11).

20

By comparing the dissolved P-alpha-1,4 glucan-binding proteins obtained by implementing the method according to the invention for identifying a protein which has an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans, with the dissolved non-phosphorylated alpha-1,4-
25 glucan-binding proteins, which are obtained, it is possible to identify proteins which have an elevated binding activity towards P-alpha-1,4 glucans compared to non-phosphorylated alpha-1,4 glucans.

In a further embodiment of the method according to the invention for identifying a
30 protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-

glucans compared to non-phosphorylated alpha-1,4 glucans, the P-alpha-1,4-glucan protein complexes obtained by incubating protein extracts with P-alpha-1,4-glucans according to step a) i and the non-phosphorylated alpha-1,4-glucan protein complexes obtained by incubating protein extracts with non-phosphorylated alpha-1,4-glucans according to step a) ii are separated from the proteins not bound to the relevant alpha-1,4-glucans. In this case, the separation takes place separately for the respective incubation solutions after process step a) i or after process step a) ii.

10 In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, the proteins dissolved according to step b) i or b) ii are separated from the alpha-1,4 glucans used in the method according to the invention according to step a) i or step a) ii.

15 In the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4 glucans, the dissolved proteins obtained according to process step b) i can either comprise a single protein or a plurality of proteins. The proteins dissolved according to process step b) ii can also either comprise a single protein or a plurality of proteins. Should the dissolved P-alpha-1,4-glucan-binding proteins or the dissolved non-phosphorylated alpha-1,4 glucan-binding proteins respectively comprise a plurality of different proteins, these are separated from one another if necessary.

25 In a further embodiment of the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans, the P-alpha-1,4-glucan-binding proteins dissolved according to process step b) i or the non-phosphorylated alpha-1,4-glucan-binding proteins dissolved according to process step b) ii are

separated from one another when implementing the method according to the invention.

The dissolved P-alpha-1,4-glucan-binding proteins or the dissolved non-phosphorylated alpha-1,4 glucan-binding proteins can be separated using methods
5 known to the person skilled in the art such as, for example, gel filtration, chromatographic methods, electrophoresis etc. The P-alpha-1,4-glucan-binding dissolved proteins or the non-phosphorylated alpha-1,4 glucan-binding dissolved proteins are preferably separated from one another by means of SDS acrylamide gel
10 electrophoresis, particularly preferably using the method described further below (see General Methods, Item 9).

A further object of the present invention is a method for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires
15 phosphorylated alpha-1,4-glucans as substrate, wherein

- a) protein extracts are incubated with phosphorylated alpha-1,4-glucans,
- b) proteins specifically bound to the phosphorylated alpha-1,4-glucans from step a) are dissolved,
- c) proteins obtained according to step b) are respectively incubated with
 - 20 i) ATP and phosphorylated alpha-1,4-glucans and
 - ii) ATP and non-phosphorylated alpha-1,4-glucansin preparations separated from one another,
- d) the respective alpha-1,4-glucan obtained after incubation in step c) i or step c) ii is examined for introduction of further phosphate groups and
- 25 e) proteins are identified which in the incubation preparation according to c) i have introduced significant quantities of phosphate groups into alpha-1,4-glucans and in the incubation preparation according to c) ii have introduced no significant quantities of phosphate groups into alpha-1,4-glucans.

The term "elevated binding activity" should be understood in conjunction with the present invention as an increased affinity of a protein to a first substrate compared to a second substrate, i.e. that the quantity of protein which under the same incubation
5 conditions binds increasedly to a first substrate compared to a second substrate, exhibits an elevated binding activity to the first substrate.

The term "alpha-1,4-glucan" should be understood in conjunction with the present invention as a glucan which mainly consists of alpha-1,4-linked glucose building
10 blocks but can also contain alpha-1,6-links as branches. An alpha-1,4-glucan preferably contains up to 15%, particularly preferably up to 10% and especially preferably up to 5% of alpha-1,6-links.

The term "starch phosphate" should be understood in conjunction with the present
15 invention as phosphate groups covalently bound to the glucose molecules of an alpha-1,4-glucan.

The term "non-phosphorylated alpha-1,4-glucan" should be understood in conjunction with the present invention as an alpha-1,4- glucan which contains no
20 detectable quantities of starch phosphate.

The term "phosphorylated alpha-1,4-glucan" or "P-alpha-1,4-glucan" should be understood in conjunction with the present invention as an alpha-1,4-glucan which contains starch phosphate.

25

Basically, a protein identifiable using a method according to the invention can come from any organism. The protein preferably comes from plant organisms, preferably from starch-storing plants (maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, *Arabidopsis*, *Curcuma* or sorghum),

particularly preferably from potato, barley, sugar beet, *Arabidopsis* or rice plants and especially preferably *Arabidopsis* or rice plants.

In a further embodiment of the method according to the invention, the protein extracts
5 come from eukaryotic cells, preferably from plant cells, particularly preferably from cells of starch-storing (maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, *arabidopsis*, curcuma or sorghum) plants.

Basically all non-phosphorylated alpha-1,4-glucans are suitable for incubating protein
10 extracts with non-phosphorylated alpha-1,4-glucans for implementing the method according to the invention. Preferably used is a non-phosphorylated plant starch, particularly preferably wheat starch and especially preferably granular leaf starch of the *Arabidopsis thaliana* mutant *sex1-3* (Tien-Shin Yu et al., 2001, Plant Cell 13, 1907-1918).

15

Methods for isolating starch from plants, for example, are known to the person skilled in the art. All methods known to the person skilled in the art are basically suitable for isolating non-phosphorylated starch from appropriate plant species. Preferably, the method for isolating non-phosphorylated alpha-1,4-glucans described below is used
20 (see General Methods Item 2)

Basically all alpha-1,4-glucans containing starch phosphate are suitable for incubating protein extracts with P-alpha-1,4-glucans for implementing the method according to the invention. Chemically phosphorylated starches can also be used in
25 this case. Preferably used for incubation with protein extracts are plant P-alpha-1,4-glucans, particularly preferably a subsequently enzymatically phosphorylated plant starch, especially preferably a subsequently enzymatically phosphorylated plant granular starch which was isolated from a *sex1-3* mutant of *Arabidopsis thaliana*.

A subsequent enzymatic phosphorylation of non-phosphorylated alpha-1,4-glucans can be carried out with any enzyme which transfers phosphate residues to non-phosphorylated alpha-1,4-glucans by introduction of covalent bonds. Preferably used for this purpose is an enzyme having the activity of a water glucan dikinase (R1 Protein, E.C.: 02.07.09.4) (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532). Preferably used for the subsequent enzymatic phosphorylation of non-phosphorylated alpha-1,4-glucans is a purified R1 protein, especially an R1 protein from potato produced by heterologous expression in *E. coli*.

10

Methods for purifying an R1 protein produced recombinantly by expression in *E. coli* are described in Ritte et al. (2002, PNAS 99, 7166-7171) and Mikkelsen et al. (2003, Biochemical Journal 377, 525-532).

15 When implementing the method according to the invention, P-alpha-1,4-glucan-protein complexes can be formed by incubation of protein extracts with P-alpha-1,4-glucans and/or non-phosphorylated alpha-1,4-glucans as a result of the binding of proteins to P-alpha-1,4-glucans and non-phosphorylated alpha-1,4-glucan-protein complexes can be formed as a result of the binding of proteins to non-phosphorylated alpha-1,4-glucans.

The proteins present in P-alpha-1,4-glucan-protein complexes or non-phosphorylated alpha-1,4-glucan-protein complexes when implementing the method according to the invention are dissolved, i.e., the binding of the proteins concerned to the respective alpha-1,4-glucans is broken. Dissolved P-alpha-1,4-glucan-binding proteins and/or dissolved non-phosphorylated alpha-1,4-glucan-binding proteins are thus obtained. Basically, all substances which prevent the existing protein-alpha-1,4-glucan interaction can be used to break the binding between the alpha-1,4-glucans concerned and the proteins bound to them. Preferred for this purpose are buffer solutions containing detergents, particularly preferably buffer solutions containing

30

sodium lauryl sulphate (SDS), especially preferably the buffer solution described further below (see General Methods Item 8).

Any method which allows alpha-1,4-glucans to be separated from the dissolved substances, such as proteins and, for example, ATP of the incubation preparation, can be used to separate alpha-1,4-glucans from ATP and/or proteins. If soluble alpha-1,4-glucans are used for the incubation of protein extracts with alpha-1,4-glucans when implementing the method according to the invention, the separation can, for example, involve a precipitation of the alpha-1,4-glucans, preferably a precipitation with suitable solvents, particularly preferably a precipitation with alcohols. The separation of alpha-1,4-glucans by binding to substances which selectively bind alpha-1,4-glucans (e.g. Concanavalin A) is also suitable for separating alpha-1,4-glucans from substances in solution.

Preferably used here for the separation of alpha-1,4-glucans is filtration, particularly preferably centrifugation, especially preferably the method described further below (see General Methods Item 8).

When implementing the method according to the invention, all methods known to the person skilled in the art, such as chromatographic methods, for example, precipitation and subsequent centrifugation of the alpha-1,4-glucan, enzymatic digestion of the alpha-1,4-glucans, gel filtration etc. which lead to separation of soluble proteins from alpha-1,4-glucans, can basically be used to separate soluble proteins from the alpha-1,4-glucans. The dissolved P-alpha-1,4-glucan-binding proteins and/or dissolved non-phosphorylated alpha-1,4-glucan-binding proteins are preferably separated from the alpha-1,4-glucans used in the method according to the invention with the aid of centrifugation.

In a further embodiment of the present invention when implementing the method according to the invention, centrifugation using a Percoll pad is used to separate P-

alpha-1,4-glucan-protein complexes from proteins not contained in the complexes concerned.

The method described further below (see General Methods Item 8) is preferably used
5 here to separate the proteins not bound to the alpha-1,4-glucans. After centrifugation
has been carried out using a Percoll pad, the proteins not bound to P-alpha-1,4-
glucans or not bound to non-phosphorylated alpha-1,4-glucans are located in the
supernatant of the centrifugation medium whereas the P-alpha-1,4-glucan-protein
complexes or non-phosphorylated alpha-1,4-glucan-protein complexes are present in
10 the sedimented pellet. The supernatant of the centrifugation medium is discarded
and the pellet is preferably washed with the buffer used for the incubation for further
purification of the P-alpha-1,4-glucan-protein complexes or non-phosphorylated
alpha-1,4-glucan-protein complexes. The pellet is preferably washed once,
particularly preferably twice.

15

Basically any type of protein extract can be used to carry out the method according to
the invention for identifying a protein which exhibits an alpha-1,4-glucan
phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as
substrate. Both so-called protein raw extracts and partly or completely purified
20 protein extracts can be involved here. Thus, for example, it is advantageous to use
proteins which were identified using a method according to the invention for
identifying a protein which exhibits an elevated binding activity towards
phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-
glucans. Proteins which were identified using a method according to the invention for
25 identifying a protein which exhibits an elevated binding activity towards
phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-
glucans, can, for example, be used omitting process steps a) and b) directly in step
c) of the method according to the invention for identifying a protein which exhibits
alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated
30 alpha-1,4-glucans as substrate.

Basically, all general methods known to the person skilled in the art, such as described, for example, in Scopes (1993, Protein Purification: Principles & Practice, ISSN: 038794072) are suitable for producing protein extracts from prokaryotic or eukaryotic cells for implementing the method according to the invention. Preferably
5 used for implementing the method, however, are methods for the isolation of plant proteins (e.g. described in Bollag et al, 1996, in: "Protein Methods", 2nd Edition, Wiley, ISBN: 0-471-11837-0; Dennison, 2003, in: "A Guide to Protein Isolation" 2nd Edition, Kluwer Academic Publishers, ISBN 1-4020-1224-1), particularly preferably the method described further below (see General Methods Item 1).

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The incubation of protein extracts for implementing the method according to the invention with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans takes place in separate preparations. The relevant preparations for P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans are treated separately from one another
15 during the implementation of the entire method. In this case, respectively the same quantities of protein extract are to be incubated with respectively the same quantities of P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans. Preferably, respectively 1 to 10 mg, particularly preferably 3 to 7 mg and especially preferably 4 to 6 mg of protein extract are incubated with P-alpha-1,4-glucans or non-
20 phosphorylated alpha-1,4-glucans. The quantity of P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans used is preferably respectively 10 to 100 mg, particularly preferably 30 to 70 mg and especially preferably 45 to 55 mg.

Various buffers can be used for the incubation of protein extracts with P-alpha-1,4-
25 glucans for implementing the method according to the invention. Basically all buffers which allow binding of the proteins to be identified to the substrate concerned are suitable. The buffer described further below (see General Methods Item 1) is preferably used.

30 The term "protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate" should be

understood in conjunction with the present invention as a protein which introduces phosphate residues covalently into P-alpha-1,4-glucans, that is uses P-alpha-1,4-glucans as a substrate for the transfer of phosphate residues whereas non-phosphorylated P-alpha-1,4-glucans are not phosphorylated by a protein concerned, i.e., non-phosphorylated P-alpha-1,4-glucans do not serve as a substrate for a phosphorylation reaction.

In a further embodiment the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method for identifying a protein which uses ATP as a further substrate.

In this embodiment of the present invention, ATP is used as a further substrate (co-substrate) by the protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, i.e. the protein concerned transfers a phosphate residue from ATP to an already phosphorylated P-alpha-1,4-glucan.

The activity of a protein which uses ATP as co-substrate for the transfer of phosphate residues to P-alpha-1,4-glucans can be demonstrated, i.e. by using ATP which contains a labeled phosphate residue (labeled ATP). To be preferred is ATP in which the phosphate residue is specifically labeled in the beta-position, i.e., in which only the phosphate residue in the beta-position has a marking. Preferably radioactively labeled ATP, particularly preferably ATP, in which the phosphate residue is specifically radioactively labeled in the beta position, and especially preferably ATP, in which the phosphate residue is specifically labeled with ^{33}P in the beta position, is used. If a P-alpha-glucan phosphorylating protein is incubated with P-alpha-1,4-glucans in the presence of labeled ATP, labeled phosphate covalently bound to the P-alpha-1,4-glucan can then be detected. In this case, the P-alpha glucans used for the phosphorylation reaction can be present both in the form of starch-phosphate-

containing plant starch (potato starch, starch from *Curcuma armada*, *C. zedoaria*, *C. longa*, rice, mung beans, tapioca etc) and also in the form of enzymatically phosphorylated P-alpha-1,4-glucans or chemically phosphorylated P-alpha-1,4-glucans. Preferably starch from leaves of *Arabidopsis thaliana*, particularly preferably starch from *Arabidopsis thaliana* *sex1-3* mutants enzymatically phosphorylated by means of an R1 protein is used.

Labeled phosphate residues which can be incorporated into a P-alpha-1,4-glucan by a protein, e.g., after separation of the labeled P-alpha-1,4-glucan (e.g., by precipitation of the alpha-1,4-glucans by means of ethanol, filtration, chromatographic methods, centrifugation etc.) from the remainder of the reaction mixture and subsequent detection of the labeled phosphate residues in the relevant P-alpha-1,4-glucan fraction, can be demonstrated. At the same time, the labeled phosphate residues bound in the P-alpha-1,4-glucan fraction can be demonstrated, for example, by determining the amount of radioactivity present in the P-alpha-1,4-glucan fraction (e.g. by means of scintillation counters).

In a further embodiment, the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method wherein the protein having alpha-1,4-glucan phosphorylating enzymatic activity uses P-starch as substrate. Starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana*, which was subsequently enzymatically phosphorylated is particularly preferred. For implementing this preferred embodiment of the method according to the invention, a phosphorylated starch is accordingly used in the process steps c) i and a non-phosphorylated starch is used in process step c) ii.

It is thereby possible to identify proteins which phosphorylate P-starch. Such proteins are especially suitable for modifying starch in plant organisms by means of genetic manipulation of appropriate plants.

In a further embodiment the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method for
5 identifying a protein wherein the protein occurs as a phosphorylated intermediate product during the transfer of a phosphate residue to a P-alpha-1,4-glucan. Said intermediate product is preferably formed by autophosphorylation of the protein concerned.

- 10 A phosphorylated protein which occurs as an intermediate product as a result of protein-mediated phosphorylation of P-alpha-1,4-glucans can be demonstrated as described in Ritte et al. (2002, PNAS 99, 7166-7171) for an R1 protein.

In order to detect the presence of an autophosphorylated intermediate product, a
15 protein is first incubated in the absence of glucans with labeled ATP, preferably with ATP labeled specifically in the beta phosphate position, particularly preferably with ATP labeled specifically with ^{33}P in the beta phosphate position for 15 to 45 minutes, particularly preferably for 20 to 40 minutes and especially preferably for 25 to 30 minutes in a reaction preparation 1. Parallel to this, a reaction preparation 2 which
20 contains corresponding quantities of non-labeled ATP instead of labeled ATP, is incubated under otherwise the same conditions. Non-labeled ATP is then added to reaction mixture 1 in excess and a mixture of non-labeled ATP and labeled ATP (the same quantity of labeled ATP as used previously in reaction mixture 1 and the same quantity of non-labeled ATP as added in excess to reaction mixture 1) is added to
25 reaction mixture 2 and incubated for a further 1 minute to 5 minutes, preferably for 2 to 5 minutes and especially preferably for 3 minutes before P-alpha-1,4-glucans are added to a Part A of reaction mixture 1 (Part 1A) or to a Part A of reaction mixture 2 (Part 2A). The reaction in the remaining Part 1B and Part 2B of the reaction mixture is stopped by denaturing the protein. Part B of the reaction mixture can be stopped
30 by the methods known to the person skilled in the art, which lead to the denaturing of proteins, preferably by adding sodium lauryl sulphate (SDS). Part 1A and Part 2A of

the reaction mixtures are incubated for at least a further 10 minutes before these reactions are also stopped. The alpha-1,4-glucans present in Part A or Part B of the respective reaction mixtures are separated from the respective remainder of the reaction mixtures. If the respective alpha-1,4-glucans are separated by centrifugation, for example, then, on completion of centrifugation, the alpha-1,4-glucans of the respective Part A or Part B of the reaction mixture are to be found in the sedimented pellet, and the proteins in the respective reaction mixtures are to be found in the supernatant of the respective centrifugation. The supernatant of Part 1A or 2A and of Part 1B or 2B of the reaction mixture can then be analysed, for example, respectively in a denaturing acrylamide gel electrophoresis, followed by autoradiography of the acrylamide gel obtained. To quantify the amount of radioactively labeled proteins, which have been separated by means of acrylamide gel electrophoresis, the so-called "phospho-imaging" method, for example, known to the person skilled in the art, can be used. If the autoradiography or the analysis by means of the "phospho-imager" of proteins in the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a protein mediating a phosphorylation of alpha-glucans occurs as an autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal in the centrifugation supernatant in the autoradiography or in the analysis by means of the "phospho-imager".

In addition, the alpha-1,4-glucans of the respective Part A of reaction mixtures 1 and 2 remaining in the respective sedimented pellet can be investigated, if necessary after subsequent washing of the respective alpha-1,4-glucans, for the presence of starch phosphate, which has a mark corresponding to the labeled ATP used. If the alpha-1,4-glucans of Part A of reaction mixture 1 contain labeled phosphate residues, and if the autoradiography of the centrifugation supernatant of Part B of reaction mixture 1 shows a significantly increased signal in the autoradiography compared with the centrifugation supernatant of Part A of reaction mixture 1, then this shows that a protein mediating a phosphorylation of alpha-glucans is present as an

autophosphorylated intermediate product. Parts A and B of reaction mixture 2 serve as a control and should therefore not exhibit a significantly increased signal for alpha-1,4-glucans labeled with ^{33}P in the sedimented pellet containing alpha-1,4-glucans.

- 5 In a further embodiment the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate relates to a method for identifying a protein which preferably introduces phosphate monoester bonds in the C-2 position or in the C-3 position, particularly preferably in the C-3 position of a
10 glucose molecule of a P-alpha-1,4-glucan.

- Which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers in the P-alpha-1,4-glucan are preferably phosphorylated by a protein or protein extract can be determined, for example, by analysing the P-alpha-1,4-glucans
15 phosphorylated by a protein or protein extract, as described in Ritte et al. (2002, PNAS 99, 7166-7171). For this purpose P-alpha-1,4-glucans additionally phosphorylated by a protein or protein extract are hydrolysed using acid and then analysed by means of anion exchange chromatography.

- 20 The P-alpha-1,4-glucans phosphorylated by a protein are preferably analysed by means of NMR in order to determine which positions of the carbon atoms (C-2, C-3 or C-6) of the glucose monomers are phosphorylated in P-alpha-1,4-glucan.

- Proteins of the method according to the invention for identifying a protein which
25 exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, which were obtained according to process step b) are incubated in step c) of the method according to the invention in separate preparations containing ATP and P-alpha-1,4-glucan or ATP and non-phosphorylated alpha-1,4-glucan. For implementing the method according to the
30 invention it is preferable to use ATP which contains a labeled phosphate residue,

particularly preferably a phosphate residue specifically labeled in the beta position, especially a phosphate residue specifically radioactively labeled in the beta position.

The incubation of dissolved proteins according to the invention with ATP and P-
5 alpha-1,4-glucans according to process step c) i or non-phosphorylated alpha-1,4-glucans according to step c) ii of the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, preferably takes place at a temperature of 20°C to 30°C, particularly preferably 23°C to 27°C and especially
10 preferably 24°C to 26°C and is carried out for a duration of at least 15 minutes, preferably for at least 20 minutes, particularly preferably for at least 30 minutes. The quantity of ATPs used in this case is preferably at least 0.05 µM, particularly preferably at least 3 µM and especially preferably at least 5 µM. The concentration of the P-alpha-1,4-glucan used or the non-phosphorylated alpha-1,4-glucan used is in
15 this case preferably at least 1 mg/ml, particularly preferably at least 10 mg/ml and especially preferably at least 25 mg/ml. After incubation has been completed, the reactions of protein extracts with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans can be stopped. The respective reaction mixture can be stopped by methods known to the person skilled in the art which lead to denaturing of proteins,
20 preferably by adding sodium lauryl sulphate and heating for 5 minutes at 95°C. When implementing step c) i or c ii) of the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, respectively the same incubation conditions for the respective incubation preparations should be carried out
25 during the incubation of proteins with P-alpha-1,4-glucans or non-phosphorylated alpha-1,4-glucans.

The P-alpha-1,4-glucan obtained according to process step c) i or the non-phosphorylated alpha-1,4-glucan obtained according to process step c) ii after
30 implementing the method according to the invention for identifying a protein which exhibits an alpha-1,4-glucan phosphorylating enzymatic activity and requires

phosphorylated alpha-1,4-glucans as substrate, is investigated for the introduction of additional phosphate residues. In order to determine whether phosphate residues were additionally introduced into the alpha-1,4-glucans concerned by process steps c) i and/or c) ii, any method which is possible for the specific detection of the marking
5 used for the labeled ATPs used in process steps c) i and c) ii can be used. If, for example, radioactively labeled ATP is used in process steps c) i or c) ii, this can be carried out using methods known to the person skilled in the art for the detection of radioactive elements, such as, for example, autoradiography, measurement of the radioactivity by means of suitable equipment (e.g. scintillation counters, "phospho-
10 imagers" etc.).

Proteins used in process step b) of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, which have
15 introduced significant quantities of phosphate residues into P-alpha-1,4-glucans in step c) i but in comparison thereto, have introduced no significant quantities of phosphate residues into non-phosphorylated alpha-1,4-glucans in step c) ii, can be identified by methods known to the person skilled in the art.

20 The term "significant quantities" should be understood in conjunction with the present invention as a quantity which is at least twice, preferably at least four times, particularly preferably at least six times and especially preferably at least eight times higher than the quantity determined in corresponding control experiments.

In this case, incubation preparations which contain completely inactivated protein
25 extracts or no protein extracts instead of native protein extracts can be used as control experiments. Protein extracts in which no more alpha-1,4-glucan phosphorylating enzymatic activity can be detected are to be understood as "completely inactivated".

Identifying proteins when implementing the method according to the invention for identifying a protein which exhibits an elevated binding activity towards phosphorylated alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans can be made using methods known to the person skilled in the art such as, for example, determining the amino acid sequence of the proteins concerned using methods comprising Edmann degradation, mass analysis using MALDI-TOF-MS (Matrix Assisted Laser Desorption/Ionization-Time Of Flight-Mass Spectroscopy), followed by comparisons with data bases containing mass profiles of proteins, amino acid sequencing by means of Q-TOF analysis or TOF/TOF analysis etc. The proteins concerned are preferably identified by means of Q-TOF-MS-MS analysis, especially preferably the proteins are identified using the method described further below (see General Methods Item 10).

If proteins are determined by means of MALDI-TOF-MS, followed by comparisons with databases containing mass profiles of proteins, the proteins concerned are first enzymatically digested beforehand before the individual masses of the protein fragments (peptides) obtained from the digestion are analysed by means of MALDI-TOF-MS. A mass profile of the protein concerned is obtained. These mass profiles are very specific for a protein since sequence-specific proteases are used for the digestion of proteins which only cleave a peptide bond when it is contained in a specific amino acid sequence succession. If the special amino acid sequence which serves as a recognition sequence for a certain protease is known, a theoretical mass profile can be created from any arbitrary amino acid sequence by calculating the mass of the peptides which would be produced after digestion of the amino acid sequence with a specific protease. By comparing mass profiles of unknown proteins actually obtained using MALDI-TOF-MS with the theoretically determined mass profiles in corresponding databases, amino acid sequences can thus also be determined.

In a further embodiment the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and

requires phosphorylated alpha-1,4-glucans as substrate, the P-alpha-1,4-glucan-protein complexes obtained by incubating protein extracts with P-alpha-1,4-glucans according to step a) are separated from the proteins not bound to the alpha-1,4-glucans concerned.

5

In a further embodiment of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, the proteins dissolved according to step b) of the method according to the invention are separated from the
10 P-alpha-1,4-glucans used in step a).

In a further embodiment the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, the dissolved P-alpha-1,4-
15 glucan-binding proteins, obtained when implementing the method according to the invention according to process step b), are separated from one another.

In a further embodiment of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and
20 requires phosphorylated alpha-1,4-glucans as substrate, the glucans obtained by incubation of protein extracts with P-alpha-1,4-glucans according to step c) i or with non-phosphorylated alpha-1,4-glucans according to step c) ii are separated from the proteins present in the reaction mixture and/or the labeled ATP present in the reaction mixture.

25

Preferably used here for the separation of alpha-1,4-glucans is filtration, particularly preferably centrifugation, especially preferably the method described further below (see General Methods Item 8). After centrifugation has been carried out using a Percoll pad, soluble substances of the reaction mixtures are located in the

supernatant of the centrifugation medium whereas the alpha-1,4-glucans are present in the sedimented pellet.

5 A further embodiment of the method according to the invention for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, relates to a method for identifying a protein which has a molecular weight derived from the amino acid sequence of 120 kDa to 145 kDa, preferably 120 kDa to 140 kDa, particularly preferably 125 kDa to 140 kDa, especially preferably 130 kDa to 135 kDa.

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In a further embodiment of the method according to the invention for identifying a protein, after identification of the proteins concerned amino acid sequences which code for these proteins are determined.

15 The amino acid sequences can be determined according to the invention using any methods known to the person skilled in the art. Such methods are sufficiently described in the specialist literature (e.g. in Protein Sequencing and Identification Using Tandem Mass Spectrometry, 2000, John Wiley & Sons Inc, ISBN: 0-471-32249-0; Protein Sequencing Protocols, 2002, Smith (Ed.), Edition: 2nd, Humana
20 Press, ISBN: 0-89603-975-7) and are basically suitable for implementing the method according to the invention. Also, the purification and/or sequencing of proteins is carried out as a contract service by many companies (e.g. Eurogentec, Searing, Belgium).

25 If necessary, proteins using a method according to the invention for identifying a protein can be subjected to further purification and/or concentration before determining their amino acid sequence. Methods for purification and/or concentration of proteins are sufficiently described in the specialist literature (e.g. in Methods in Enzymology: Guide to Protein Purification, Vol.182 1990, Deutscher, Murray P. (Ed.),
30 Academic Press, ISBN: 0-12-182083-1; Isolation and Purification of Proteins: Hatti-

Kaul, 2003, Rajni (Ed.); Mattiasson, Bo (Edt), Marcel Dekker Inc, ISBN:0-8247-0726-5, Protein Purification Techniques: A Practical Approach. Roe, 2001, Simon (Ed.). The Practical Approach Series, 244. Edition: 2nd. Oxford Univ Press, ISBN: 0-19-963673-7) and are basically suitable for implementing the method according to the
5 invention.

In a further embodiment of the method according to the invention for identifying a protein, methods according to the invention for identifying a protein whose encoding amino acid sequence has a phosphohistidine domain (Tien-Shin Yu et al., 2001,
10 Plant Cell 13, 1907-1918) are used. The phosphohistidine domain preferably has an identity of at least 50% with the amino acid sequence of the phosphohistidine domain of the OK1 protein from *Arabidopsis thaliana* and *Oryza sativa* specified under SEQ ID NO 5, in particular of at least 60%, preferably of at least 70% and particularly preferably of at least 80% and especially preferably of at least 90%.

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In a further embodiment of the method according to the invention for identifying a protein, methods according to the invention for identifying a protein whose encoding amino acid sequence has a phosphohistidine domain (Tien-Shin Yu et al., 2001, Plant Cell 13, 1907-1918) are used wherein the phosphohistidine domain contains
20 two histidines.

Using the methods according to the invention, proteins which exhibit an elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans can be identified.

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Using the method according to the invention, proteins which exhibit alpha-1,4-glucan phosphorylating enzymatic activity and require phosphorylated alpha-1,4-glucans as substrate can be identified.

Thus, proteins obtainable by methods according to the invention for identifying a protein are also the object of the present invention.

5 A method for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity is further an object of the present invention, wherein

- a) a protein is identified using a method according to the invention for identifying a protein,
- b) amino acid sequences coding for the protein identified according to step
10 a) are determined and
- c) nucleic acids are identified using the amino acids determined according to step b), which code for a protein identified according to step a).

15 The amino acid sequence of the proteins identified using a method according to the invention can be determined using methods known to the person skilled in the art, as already stated above.

20 On the basis of the amino acid sequences determined according to step b) of the method according to the invention for identifying a nucleic acid, coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, nucleic acids coding for a protein exhibiting alpha-1,4-glucan-phosphorylating enzymatic activity can be identified.

25 Nucleic acids coding for a protein exhibiting alpha-1,4-glucan-phosphorylating enzymatic activity can be identified, for example, by scrutinising databases such as those made available, for example by EMBL (<http://www.ebi.ac.uk/Tools/index.htm>) or NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>). In this case one or a plurality of amino acid sequences determined when implementing the method according to the invention, is pre-defined

as a so-called query. This query sequence is then compared by means of statistical computer programs with sequences, which are contained in the selected databases. Such database queries (e.g. blast or fasta searches) are known to the person skilled in the art and can be carried out by various providers.

5

If such a database query is carried out, e.g. at the NCBI (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), then the standard settings, which are specified for the particular comparison inquiry, should be used. For protein sequence comparisons (blastp), these are the following settings: Limit entrez = not
10 activated; Filter = low complexity activated; Expect value = 10; word size = 3; Matrix = BLOSUM62; Gap costs: Existence = 11, Extension = 1.

During such a database search, for example, the amino acid sequences determined in the present invention when implementing the method according to the invention
15 can be used as a query sequence in order to identify nucleic acid molecules coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity.

Using the method described, it is also possible to identify nucleic acid molecules and/or amino acid sequences which have a high degree of identity to nucleic acid molecules and/or proteins obtainable using the method according to the invention
20 and coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity.

Methods are known to the person skilled in the art with which, starting from amino acid sequences, he can identify nucleic acids coding for these (see, for example,
25 Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd edition (2001) Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. ISBN: 0879695773, Ausubel et al., Short Protocols in Molecular Biology, John Wiley & Sons; 5th edition (2002), ISBN: 0471250929). From amino acid sequences coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity, nucleic acids coding for
30 the amino acid sequences concerned can be derived in accordance with the genetic

code. It is known to the person skilled in the art that the degenerated oligonucleotides obtained from the genetic code can basically also be used to identify nucleic acids. Oligonucleotides which constitute sequences derived from the amino acid sequences obtained when implementing the method according to the invention can then be synthesised. These synthetic oligonucleotides can be used to identify nucleic acids coding for the proteins from whose amino acid sequence the corresponding oligonucleotide sequences were derived. This can be achieved, for example, by searching gene libraries, said synthetic oligonucleotides being used as labeled probes in the form of hybridisation probes. A further possibility for identifying nucleic acids coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity involves using the synthetic oligonucleotides derived from amino acid sequences obtained when implementing the method according to the invention, by searching gene libraries using PCR based methods, wherein said synthetic oligonucleotides are used as so-called "primers". Gene libraries can be present, for example, in the form of cosmids, phagmids, plasmids, YACs or BACs. The DNA libraries can contain both genomic and also cDNA. For PCR-based searching methods when using the so-called RT (Reverse Transcription) PCR, it is also possible to use mRNA. The nucleic acids for the implementation of the method according to the invention for identifying a nucleic acid in gene libraries or present as mRNA can in this case come from any organism, preferably they come from eukaryotic, particularly preferably from plants, especially preferably from cereals.

For the implementation of the method according to the invention for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, it is not necessary that the entire amino acid sequence coding for the protein concerned is determined in step b) of the method according to the invention but it can be sufficient if only parts of the amino acid sequences concerned, coding for a protein concerned, are determined.

A further embodiment of the present invention relates to a method for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, wherein

- 5 a) a protein is identified using a method according to the invention for identifying a protein,
- b) amino acid sequences coding for the protein identified according to step a) are determined
- c) oligonucleotides are synthesised starting from the amino acid sequences determined in step b) and
- 10 d) nucleic acids coding for a protein identified according to step a) are identified with the aid of the oligonucleotides synthesised according to step c)

A further object of the present invention relates to a method for identifying a nucleic acid coding for a protein which exhibits alpha-1,4-glucan-phosphorylating enzymatic activity, wherein

- 15 a) a protein is identified using a method according to the invention for identifying a protein,
- b) antibodies which react specifically with the protein identified according to step a) are produced and
- 20 c) nucleic acids are identified using the antibodies determined according to step b).

Methods for manufacturing antibodies, which react specifically with a certain protein, i.e. which bind specifically to said protein, are known to the person skilled in the art
25 (see, for example, Lottspeich and Zorbas (Eds.), 1998, Bioanalytik, Spektrum akad, Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4). The manufacture of such antibodies is offered by some companies (e.g. Eurogentec, Belgium) as a contract service.

Methods for identifying nucleic acids using antibodies, frequently designated as "immunoscreening" in the specialist literature (see, for example Lottspeich und Zorbas (Eds.), 1998, Bioanalytik, Spektrum akademischer Verlag, Heidelberg, Berlin, ISBN 3-8274-0041-4) are likewise known to the person skilled in the art and described in detail in the literature. So-called expression gene libraries, for example, can be used to implement such methods, in which the clones obtained are screened for the expression of a certain protein with the aid of a specific antibody directed against this protein. Materials for manufacturing such expression gene libraries, also containing instructions relating the method for the manufacture and also methods for searching such expression gene banks can be purchased (e.g. Stratagene).

Using methods according to the invention, it is possible to identify nucleic acids coding for proteins which exhibit elevated binding activity towards P-alpha-1,4-glucans compared to non-phosphorylated alpha-1,4-glucans and/or which exhibit alpha-1,4-glucan phosphorylating enzymatic activity and require phosphorylated alpha-1,4-glucans as substrate.

Thus, nucleic acids obtainable by methods according to the invention for identifying a nucleic acid, are also the object of the present invention.

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A plasmid (A.t.-OK1-pGEM) containing a cDNA which codes for a protein according to the invention (A.t.-OK1) from *Arabidopsis thaliana* was deposited on 08.03.2004 under the number DSM16264 and a plasmid (pM150) containing a cDNA which codes for further protein according to the invention (O.s.-OK1) from *Oryza sativa* was deposited on 24.03.2004 under the number DSM16302 under the Budapest Treaty at the German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany.

It was surprisingly found that genetically modified plant cells or plants which exhibit an elevated activity of a protein according to the invention, synthesise a modified

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starch which is modified in its physical-chemical properties, especially the content of starch phosphate or the phosphate distribution compared to starch synthesised in wild type plant cells or wild type plants so that this is better suited for special applications.

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Thus, a further object of the present invention relates to genetically modified plant cells or genetically modified plants characterised in that they exhibit an elevated enzymatic activity of a protein according to the invention compared to corresponding non-genetically modified wild type plant cells or wild type plants.

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In this case, the genetic modification can be any genetic modification, which leads to an increase in the activity of at least one protein according to the invention in comparison with corresponding wild type plant cells or wild type plants that have not been genetically modified.

15

In conjunction with the present invention, the term "wild type plant cell" means that the plant cells concerned were used as starting material for the manufacture of the plant cells according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant cell according to the invention.

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In conjunction with the present invention, the term "wild type plant" means that the plants concerned were used as starting material for the manufacture of the plants according to the invention, i.e. their genetic information, apart from the introduced genetic modification, corresponds to that of a plant according to the invention.

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In conjunction with the present invention, the term "corresponding" means that, in the comparison of several objects, the objects concerned that are compared with one another have been kept under the same conditions. In conjunction with the present

invention, the term "corresponding" in conjunction with wild type plant cell or wild type plant means that the plant cells or plants, which are compared with one another, have been raised under the same cultivation conditions and that they have the same (cultivation) age.

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The term "elevated activity" in the framework of the present invention means in this case an increase in the expression of endogenous genes coding for proteins according to the invention and/or an increase in the quantity of proteins according to the invention in the cells and/or an increase in the enzymatic activity of proteins according to the invention in the cells.

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The increase in the expression can, for example, be determined by measuring the quantity of transcripts coding for proteins according to the invention, e.g. using Northern blot analysis or RT-PCR. Nucleic acid molecules which were identified using methods according to the invention for identifying a nucleic acid are preferably used in this case to determine an elevated expression of proteins according to the invention. Here, an increase preferably means an increase in the amount of transcripts in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase in the quantity of transcripts coding for a protein according to the invention also means that plants which have no detectable transcripts coding for a protein according to the invention, after genetic modification according to the invention, have a detectable quantity of transcripts coding for a protein according to the invention.

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The increase in the amount of protein of a protein according to the invention, which results in an increased activity of this protein in the plant cells concerned, can, for example, be determined by immunological methods such as Western Blot analysis, ELISA (Enzyme Linked Immuno Sorbent Assay) or RIA (Radio ImmunoAssay). The manufacture of an antibody which can be used to measure the increase in the amount of protein using immunological methods is described further below as an

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example (see Example 11). Here, an increase preferably means an increase in the amount of a protein according to the invention in comparison with corresponding cells that have not been genetically modified by at least 50%, in particular by at least 70%, preferably by at least 85% and particularly preferably by at least 100%. An increase
5 in the amount of a protein according to the invention also means that plants which have no detectable amount of a protein according to the invention, after genetic modification according to the invention, have a detectable amount of protein according to the invention.

10 It was surprisingly also found that genetically modified plant cells or plants which exhibit a reduced activity of a protein according to the invention, synthesise a modified starch which is modified in its physical-chemical properties, especially relating to the phosphate distribution compared to starch synthesised in wild type plant cells or wild type plants so that this is better suited for special applications.

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Thus, a further object of the present invention relates to genetically modified plant cells or genetically modified plants, characterised in that they exhibit a reduced enzymatic activity of a protein according to the invention compared to corresponding wild type plant cells or wild type plants which have not been genetically modified .

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Plants which exhibit a reduced activity of a protein according to the invention, exhibit a high starch (starch excess) phenotype. Furthermore, plants which exhibit a reduced activity of a protein according to the invention, exhibit normal growth compared to wild type plants, i.e., the plants are not hindered in their growth by the
25 reduced activity of a protein according to the invention. Therefore, plants which exhibit a reduced activity of a protein according to the invention, are suitable for cultivation in agriculture since they contain more starch and therefore more carbohydrate and at the same time show no reduction in growth rate.

The present invention therefore also relates to plant cells and plants according to the invention which exhibit a starch excess phenotype. Plant cells according to the invention and plants according to the invention have at least twice, preferably at least four times, particularly preferably at least six times and especially preferably at least eight times more starch in their leaves at the end of the dark phase than corresponding wild type plant cells or wild type plants.

Plant cells according to the invention and plants according to the invention have at least 1.2 times, preferably at least 1.5 times, particularly preferably at least 1.8 times and especially preferably at least twice more starch in their leaves at the end of the light phase than corresponding wild type plant cells or wild type plants.

The plant cells according to the invention and plants according to the invention which exhibit a reduced activity of a protein according to the invention, can be manufactured by various methods known to the person skilled in the art. These include, for example, the expression of a corresponding antisense RNA, or a double-stranded RNA construct, the preparation of molecules or vectors which impart a co-suppression effect, the expression of a correspondingly constructed ribozyme which specifically cleaves transcripts which code for a protein according to the invention or the so-called "*in vivo* mutagenesis". Moreover, the reduction of the activity of a protein according to the invention in plant cells and plants can also be brought about by the simultaneous expression of sense and antisense RNA molecules of the respective target gene to be repressed, preferably the OK1 gene.

It is additionally known that the *in planta* formation of double-stranded RNA molecules of promoter sequences *in trans* can lead to a methylation and a transcriptional inactivation of homologous copies of this promoter (Mette et al., EMBO J. 19, (2000), 5194-5201).

Another possible method for reducing the enzymatic activity of proteins in plant cells or plants is the so-called immunomodulation method. It is known that an *in planta* expression of antibodies which specifically recognise a plant protein results in a reduction in the activity of the relevant protein in corresponding plant cells as a result of the formation of a protein antibody complex (Conrad and Manteufel, Trends in Plant Science 6, (2001), 399-402; De Jaeger et al., Plant Molecular Biology 43, (2000), 419-428; Jobling et al., Nature Biotechnology 21, (2003), 77-80).

All these methods are based on the introduction of a foreign or a plurality of foreign nucleic acid molecules into the genome of plant cells or plants and are therefore fundamentally suitable for manufacturing plant cells according to the invention and plants according to the invention.

In a further embodiment of the present invention, the plant cells according to the invention or plants according to the invention comprise plant cells of starch-storing plants or starch-storing plants. Starch-storing plants are, for example, maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, *Arabidopsis*, curcuma or sorghum plants. Particularly preferred are rice, especially preferred are wheat plants.

A further embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant.

In this context, the term "genetic modification" means the introduction of homologous and/or heterologous foreign nucleic acid molecules into the genome of a plant cell or into the genome of a plant, wherein said introduction of these molecules leads to an increase or reduction in the activity of a protein according to the invention.

The plant cells according to the invention or plants according to the invention are modified with regard to their genetic information by the introduction of a foreign nucleic acid molecule. The presence or the expression of the foreign nucleic acid molecule leads to a phenotypic change. "Phenotypic" change preferably means in
5 this case a measurable change in one or a plurality of functions of the cells. For example, the genetically modified plant cells according to the invention and the genetically modified plants according to the invention exhibit an increase or reduction in the activity of a protein according to the invention due to the presence or on the expression of the introduced nucleic acid molecule.

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In conjunction with the present invention, the term "foreign nucleic acid molecule" is understood to mean such a molecule that either does not occur naturally in the corresponding wild type plant cells, or that does not occur naturally in the specific spatial arrangement in wild type plant cells, or that is localised at a place in the
15 genome of the wild type plant cell at which it does not occur naturally. Preferably, the foreign nucleic acid molecule is a recombinant molecule, which consists of different elements, the combination or specific spatial arrangement of which does not occur naturally in plant cells.

20 In principle, the foreign nucleic acid molecule can be any nucleic acid molecule, which effects an increase in the activity of a protein according to the invention in the plant cell or plant.

In conjunction with the present invention, the term "genome" is to be understood to
25 mean the totality of the genetic material present in a plant cell. It is known to the person skilled in the art that, as well as the cell nucleus, other compartments (e.g. plastids, mitochondria) also contain genetic material.

A preferred embodiment of the present invention relates to a genetically modified
30 plant cell according to the invention or a genetically modified plant according to the

invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant and the foreign nucleic acid molecule codes for a protein according to the invention.

- 5 A further embodiment of the present invention relates to a genetically modified plant cell according to the invention or a genetically modified plant according to the invention, wherein the genetic modification consists in the introduction of at least one foreign nucleic acid molecule into the genome of the plant and wherein the foreign nucleic acid molecule comprises a nucleic acid molecule according to the invention,
10 preferably a nucleic acid molecule according to the invention, isolated from *Arabidopsis thaliana*, particularly preferably isolated from rice.

- A large number of techniques are available for the introduction of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA
15 using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation medium, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA by means of the biolistic approach as well as other possibilities.

- The use of agrobacteria-mediated transformation of plant cells has been intensively
20 investigated and adequately described in EP 120516; Hoekema, in: The Binary Plant Vector System Offsetdrukkerij Kanter B.V., Alblaserdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant Sci. 4, 1-46 and by An et al. EMBO J. 4, (1985), 277-287. For the transformation of potato, see Rocha-Sosa et al., EMBO J. 8, (1989), 29-33, for example.

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- The transformation of monocotyledonous plants by means of vectors based on *Agrobacterium* transformation has also been described (Chan et al., Plant Mol. Biol. 22, (1993), 491-506; Hiei et al., Plant J. 6, (1994) 271-282; Deng et al, Science in China 33, (1990), 28-34; Wilmink et al., Plant Cell Reports 11, (1992), 76-80; May et al., Bio/Technology 13, (1995), 486-492; Conner and Domisse, Int. J. Plant Sci. 153
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(1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). 153 (1992), 550-555; Ritchie et al, Transgenic Res. 2, (1993), 252-265). An alternative system to the transformation of monocotyledonous plants is transformation by means of the biolistic approach (Wan and Lemaux, Plant Physiol. 104, (1994), 37-48; Vasil et al., 5 Bio/Technology 11 (1993), 1553-1558; Ritala et al., Plant Mol. Biol. 24, (1994), 317-325; Spencer et al., Theor. Appl. Genet. 79, (1990), 625-631), protoplast transformation, electroporation of partially permeabilised cells and the introduction of DNA by means of glass fibres. In particular, the transformation of maize has been described in the literature many times (cf. e.g. WO95/06128, EP0513849, 10 EP0465875, EP0292435; Fromm et al., Biotechnology 8, (1990), 833-844; Gordon-Kamm et al., Plant Cell 2, (1990), 603-618; Koziel et al., Biotechnology 11 (1993), 194-200; Moroc et al., Theor. Appl. Genet. 80, (1990), 721-726).

The successful transformation of other types of cereal has also already been 15 described, for example, for barley (Wan and Lemaux, see above; Ritala et al., see above; Krens et al., Nature 296, (1982), 72-74) and for wheat (Nehra et al., Plant J. 5, (1994), 285-297). All the above methods are suitable within the framework of the present invention.

20 Amongst other things, the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells and wild type plants respectively in that they contain a foreign nucleic acid molecule, which does not occur naturally in wild type plant cells or wild type plants, or in that such a molecule is present integrated at a place in the genome of the plant cell according to 25 the invention or in the genome of the plant according to the invention at which it does not occur in wild type plant cells or wild type plants, i.e. in a different genomic environment. Furthermore, plant cells according to the invention and plants according to the invention of this type differ from wild type plant cells and wild type plants respectively in that they contain at least one copy of the foreign nucleic acid molecule 30 stably integrated within their genome, possibly in addition to naturally occurring copies of such a molecule in the wild type plant cells or wild type plants. If the foreign

nucleic acid molecule(s) introduced into the plant cells according to the invention or into the plants according to the invention is (are) additional copies of molecules already occurring naturally in the wild type plant cells or wild type plants respectively, then the plant cells according to the invention and the plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively in particular in that this additional copy or these additional copies is (are) localised at places in the genome at which it does not occur (or they do not occur) in wild type plant cells or wild type plants. This can be verified, for example, by using a Southern blot analysis.

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Furthermore, the plant cells according to the invention and plants according to the invention can be differentiated from wild type plant cells or wild type plants respectively preferably by at least one of the following features: If the foreign nucleic acid molecule that has been introduced is heterologous with respect to the plant cell or plant, then the plant cells according to the invention or plants according to the invention have transcripts of the introduced nucleic acid molecules. These can be verified, for example, by Northern blot analysis or by RT-PCR (Reverse Transcription Polymerase Chain Reaction). Preferably, the plant cells according to the invention and the plants according to the invention which exhibit an elevated activity of a protein according to the invention, contain a protein, which is coded for by an introduced nucleic acid molecule. This can be demonstrated by immunological methods, for example, in particular by a Western blot analysis. Plant cells according to the invention and plants according to the invention which exhibit a reduced activity of a protein according to the invention, show a reduced quantity of the relevant protein compared to corresponding wild type plant cells or wild type plants which have not been genetically modified, when investigated using said immunological methods.

If the foreign nucleic acid molecule that has been introduced is homologous with respect to the plant cell or plant, the plant cells according to the invention or plants according to the invention can be differentiated from wild type plant cells or wild type

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plants respectively due to the additional expression of the introduced foreign nucleic acid molecule, for example. The plant cells according to the invention and the plants according to the invention preferably contain transcripts of the foreign nucleic acid molecules. This can be demonstrated by Northern blot analysis, for example, or
5 using so-called quantitative PCR.

In a special embodiment, the plant cells according to the invention and the plants according to the invention are transgenic plant cells or transgenic plants respectively

10 In a further embodiment of the present invention, plant cells according to the invention and plants according to the invention synthesise a modified starch compared to starch isolated from wild type plant cells or wild type plants which have not been genetically modified.

15 In conjunction with the present invention, the term "modified starch" means that the starch has changed physical-chemical characteristics compared with non-modified starch obtainable from corresponding wild type plant cells or wild type plants.

In a further embodiment, the plant cells according to the invention or the plants
20 according to the invention synthesise a modified starch which has an elevated content of starch phosphate and/or a modified phosphate distribution compared with starch isolated from corresponding wild type plant cells or wild type plants.

In a further embodiment of the method according to the present invention, the plant
25 cells according to the invention or the plants according to the invention synthesise a modified starch which has a modified C-3/C-6 ratio of the starch phosphate compared with corresponding wild type plants cells which have not been genetically modified or plants which have not been genetically modified. Especially preferred in this case are starches which exhibit an elevated fraction of starch phosphate bound

in the C-3 position compared with starch phosphate bound in the C-6 position, in comparison to corresponding starches isolated from wild type plant cells which have not been genetically modified or plants which have not been genetically modified.

5 In conjunction with the present invention, the term "phosphate distribution" should be understood as the fraction of the starch phosphate bound in the C-2 position, C-3 position or C-6 position of a glucose molecule relative to the total starch phosphate content of alpha-1,4-glucans.

10 In conjunction with the present invention, the term "C-2/C-3/C-6 ratio" should be understood as the fraction of the starch phosphate in which the starch phosphate of an alpha-1,4-glucan bound respectively in the C-2 position, C-3 position or C-6 position contributes to the total starch phosphate content of the alpha-1,4-glucan concerned (C-2 position + C-3 position + C-6 position).

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In conjunction with the present invention, the term "C-3/C-6 ratio" should be understood as the fraction of the starch phosphate in which the starch phosphate of an alpha-1,4-glucan bound respectively in the C-3 position and in the C-6 position contributes to the sum of the starch phosphate bound in the C-3 position and in the

20 C-6 position (C-3 position + C-6 position) of the alpha-1,4-glucan concerned.

A further object of the present invention is plant cells according to the invention or plants according to the invention which synthesise a modified starch, wherein the modified starch is characterised in that it has an elevated content of phosphate covalently bound to the starch in the C-3 position of the glucose molecule compared to starch from corresponding wild type plant cells or wild type plants.

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A further object of the present invention is plants containing plant cells according to the invention.

Description of sequences

SEQ ID NO 1: Nucleic acid sequence containing the coding region of the A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence is inserted in the vectors
5 OK1-pGEM-T and OK1-pDEST™17.

SEQ ID NO 2: Amino acid sequence coding for the A.t.-OK1 protein from *Arabidopsis thaliana*. This sequence can be derived from the nucleic acid sequence shown under SEQ ID NO 1.

SEQ ID NO 3: Nucleic acid sequence containing the coding region of the O.s.-
10 OK1 protein from *Oryza sativa*. This sequence is inserted in the vector MI50.

SEQ ID NO 4: Amino acid sequence coding for the O.s.-OK1 protein from *Oryza sativa*. This sequence can be derived from the nucleic acid sequence shown under SEQ ID NO 3.

SEQ ID NO 5: Peptide sequence coding for the phosphohistidine domain of the
15 OK1 proteins from *Arabidopsis thaliana*, *Oryza sativa* and *Sorghum bicolor*.

SEQ ID NO 6: Peptide sequence contained in the amino acid sequence coding for an H.v.-OK1 protein from barley.

SEQ ID NO 7: Peptide sequence contained in the amino acid sequence coding for an H.v.-OK1 protein from barley.

20 SEQ ID NO 8: Peptide sequence contained in the amino acid sequence coding for an H.v.-OK1 protein from barley.

SEQ ID NO 9: Partial nucleic acid sequence coding for an H.v.-OK1 protein from barley. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 6, SEQ ID NO 7 and SEQ ID NO 8 using the
25 "Blast search" facility in the TIGR database.

SEQ ID NO 10: Partial amino acid sequence coding for an H.v.-OK1 protein from barley. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 9.

SEQ ID NO 11: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

SEQ ID NO 12: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

5 SEQ ID NO 13: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

SEQ ID NO 14: Peptide sequence contained in the amino acid sequence coding for an S.t.-OK1 protein from potato.

10 SEQ ID NO 15: Partial nucleic acid sequence coding an S.t.-OK1 protein from potato. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13 and SEQ ID NO 14 using the "Blast Search" facility in the TIGR database.

15 SEQ ID NO 16: Partial amino acid sequence coding for an S.t.-OK1 protein from potato. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 15.

SEQ ID NO 17: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

SEQ ID NO 18: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

20 SEQ ID NO 19: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

SEQ ID NO 20: Peptide sequence contained in the amino acid sequence coding for an S.b.-OK1 protein from millet.

25 SEQ ID NO 21: Partial nucleic acid sequence coding for an S.b.-OK1 protein from millet. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19 and SEQ ID NO 20 using the "Blast Search" facility in the TIGR database.

SEQ ID NO 22: Partial amino acid sequence coding for an S.b.-OK1 protein from millet. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 21.

5 SEQ ID NO 23: Peptide sequence contained in the amino acid sequence coding for a T.a.-OK1 protein from wheat.

SEQ ID NO 24: Peptide sequence containing the amino acid sequence coding for a T.a.-OK1 protein from wheat.

10 SEQ ID NO 25: Partial nucleic acid sequence coding for a T.a.-OK1 protein from wheat. This nucleic acid sequence has been identified by means of the peptide sequences shown under SEQ ID NO 23 and SEQ ID NO 24 using the "Blast Search" facility in the TIGR database.

SEQ ID NO 26: Partial amino acid sequence coding for a T.a.-OK1 protein from wheat. The amino acid sequence shown can be derived from the nucleic acid sequence shown under SEQ ID NO 25.

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Description of the Figures

Fig. 1: Denaturing acrylamide gel for identifying proteins from *Arabidopsis thaliana*, which preferably bind to non-phosphorylated starch in comparison with phosphorylated starch. A standard protein molecular weight marker is shown in trace "M". Proteins obtained after incubating control preparation C from Example 1 d) are shown in trace "-". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with non-phosphorylated starch, isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant (Preparation B, Example 1 d)), are shown in trace "K". Protein extracts of *Arabidopsis thaliana*, obtained after incubation with starch, isolated from leaves of an *Arabidopsis thaliana* *sex1-3* which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation A, Example 1 d), are shown in trace "P". On completion of electrophoresis, the acrylamide gel was stained with Coomassie Blue.

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Fig. 2: Demonstration of the autophosphorylating activity of the OK1 protein. Fig. 2A) shows a denaturing (SDS) acrylamide gel stained with Coomassie Blue on completion of the electrophoresis. Figure 2 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. M: Standard protein molecular weight marker; R1: Sample from reaction vessel 1 according to Example 7 (after incubating an OK1 protein with ATP); R2: Sample from reaction vessel 2 according to Example 7 (after incubating an OK1 protein with ATP the protein was heated to 95°C); R3: Sample from reaction vessel 3 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M HCl); R4: Sample from reaction vessel 4 according to Example 7 (after incubating an OK1 protein with ATP the protein was incubated in 0.5 M NaOH).

Fig. 3: Demonstration of the starch-phosphorylating activity of an OK1 protein (see Example 6). OK1 protein was incubated with non-phosphorylated starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant (Preparation A) and starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1 protein (Preparation B). Preparation C is the same as Preparation B, except that this Preparation C was incubated without OK1 protein. Two independent tests were carried out for each preparation (A, B, C) (Test 1 and Test 2). The respective amounts are shown, measured in cpm (counts per minute), on ³³P labeled phosphate, which was introduced into non-phosphorylated starch (Preparation A) and phosphorylated starch (Preparation B).

Fig. 4: Comparison of the C-atom positions of glucose molecules of the starch, which was phosphorylated from an R1 protein and an OK1 protein respectively (see Example 9). OK1 protein (Preparation A) was incubated in the presence of ATP labeled with ³³P with starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, which was phosphorylated retrospectively *in vitro* with an R1

protein.). R1 protein (preparation B) was incubated in the presence of ATP labeled with ^{33}P with starch isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant. After incubation had been completed, a total hydrolysis of the starch was carried out and the hydrolysis products obtained were separated using HPAE chromatography.

5 As standard, glucose-6-phosphate and glucose-3-phosphate were added to the hydrolysis products before separation. The hydrolysis products separated by means of HPAE chromatography were collected in individual fractions. The added glucose-6-phosphate eluted with fraction 15 and the added glucose-3-phosphate with fraction 17. The fractions obtained were subsequently investigated for the presence of

10 radioactively labeled phosphate. The amount of ^{33}P labeled phosphate measured in the individual fractions, measured in cpm (counts per minute), which was introduced into the hydrolysis products of the phosphorylated starch by the OK1 protein or the R1 protein, is shown graphically.

15 Fig. 5 Demonstration of the autophosphorylation of the OK1 protein. Figure 5 A) shows a Western Blot. Figure 5 B) shows the autoradiography of a denaturing (SDS) acrylamide gel. The same amounts of the same samples were applied to each of the two gels. The OK1 protein was incubated either with randomised radioactively labeled ATP or with ATP specifically radioactively labeled in

20 the gamma position. On completion of incubation, the proteins were either heated to 30°C or 95°C, or incubated in 0.5 M NaOH or 0.5 M HCl respectively.

Fig. 6 Demonstration of the transfer of the beta-phosphate residue of ATP to starch in a reaction catalysed by an OK1 protein. Either ATP specifically

25 labeled with ^{33}P in the gamma position or randomised ^{33}P ATP was used to phosphorylate starch, which had been phosphorylated *in vitro* by means of an R1 protein and isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, by means of an OK1 protein. No OK1 protein was added in any of the experiments designated as "control". Each preparation was tested twice, independently of one another. The

30 results of both tests are shown.

Fig. 7 Western Blot analysis of protein extracts from plants using an antibody against the OK1 protein from *Arabidopsis thaliana*. Protein extracts from leaves of the following plants are shown: Ara, *Arabidopsis thaliana*; 51, 54, 55, 67, 72, 73, 79, 62, 63, 64, 65, 69, 66, 68 are independent lines of the transformation 385JH; D
5 wildtype *Solanum tuberosum* cv Désirée.

General methods

10 In the following, methods are described, which can be used for carrying out the method according to the invention. These methods constitute specific embodiments of the present invention but do not restrict the present invention to these methods. The person skilled in the art knows that he can implement the invention in the same way by modifying the methods described and/or by replacing individual parts of the
15 methods by alternative parts of the methods.

1. Manufacture of protein extracts from plant tissue

a) Manufacture of protein extracts from plant tissues

Leaf material is frozen in liquid nitrogen immediately after harvesting and
20 subsequently homogenised in the mortar under liquid nitrogen. The reduced leaf material is mixed with ca. 3.5 times the volume (relative to the weight of the leaf material used) of cold (4°C) binding buffer and macerated for 2x10 s using an Ultraturrax (maximum speed). After the first treatment with an Ultraturrax, the reduced leaf material is cooled on ice before the second treatment is carried out. The
25 treated leaf material is then passed through a 100 µm nylon mesh and centrifuged for 20 min (50 ml centrifuge vessel, 20,000xg, 4°C).

b) Precipitation of the proteins contained in the protein extracts

The supernatant obtained following centrifugation according to Step a) is removed and its volume determined. To precipitate proteins, ammonium sulphate is added continuously to the supernatant over a period of 30 minutes while stirring on ice down to a final concentration of 75% (weight/volume). The supernatant is subsequently
5 incubated for a further hour on ice while stirring. The proteins precipitated from the supernatant are pelleted at 20,000xg and 4°C for 10 min and the pellet subsequently absorbed in 5 ml of binding buffer, i.e. the proteins present in the pellet are dissolved.

10 c) Desalting of the precipitated proteins

The dissolved proteins are desalted using a PD10 column filled with Sephadex G25 (Amersham Bioscience, Freiburg, Prod. No. columns: 17-0851-01, Prod. No. Sephadex G25-M: 17-0033-01) at a temperature of 4°C, i.e. the ammonium sulphate used for the precipitation under step b) is separated from the dissolved proteins. The
15 PD10 column is equilibrated with binding buffer before the proteins dissolved in accordance with Step b) are applied. For this purpose, 5 ml of binding buffer are spread over the column in each case. Subsequently, 2.5 ml of the protein solution obtained in accordance with Step b) are added to each column before proteins are eluted from the column with 3.5 ml binding buffer.

20

d) Determination of the protein concentration

The protein concentration is determined with a Bradford assay (Biorad, Munich, Prod. No. 500-0006 (Bradford, 1976, Anal. Biochem. 72, 248-254)).

25 e) Composition of the binding buffer [

Binding buffer:	50 mM	HEPES/NaOH (or KOH), pH 7.2
	1 mM	EDTA
	2 mM	Dithioerythritol (DTE)
	2 mM	Benzamidine

- 51 -

2 mM	ϵ -aminocaproic acid
0.5 mM	PMSF
0.02 %	Triton X-100

2. Isolation of leaf starch

5 a) Isolation of starch granules from plant tissues

Leaf material is frozen immediately after harvesting in liquid nitrogen. The leaf material is homogenised in portions in the mortar under liquid nitrogen and absorbed into a total of ca. 2.5-times the volume (weight/volume) of starch buffer. In addition, this suspension is again homogenised in a Waring blender for 20 s at maximum speed. The homogenate is passed through a nylon mesh (100 μ m mesh width) and centrifuged for 5 minutes at 1,000xg. The supernatant with the soluble proteins is discarded.

b) Purifying the starch isolated from the plant tissues

15 After removing the green material lying on top of the starch by rinsing off the green material with starch buffer, the pellet containing the starch obtained from Step a) is absorbed in starch buffer and successively passed through nylon meshes with different mesh widths (in the order 60 μ m, 30 μ m, 20 μ m). The filtrate is centrifuged using a 10 ml Percoll cushion (95% (v/v) Percoll (Pharmacia, Uppsala, Sweden), 5% (v/v) 0.5M HEPES-KOH pH7.2) (Correx tube, 15 min, 2,000xg). The sediment
20 obtained after this centrifugation is resuspended once in starch buffer and centrifuged again (5 min, 1,000xg).

c) Removal of the proteins bound to the starch

25 Following Step b), starch granules are obtained, which contain proteins bound to the starch. The proteins bound to the surface of the starch granules are removed by incubating four times with 0.5 % SDS (sodium lauryl sulphate) for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a

centrifugation (5 min, 5,000xg), in order to separate the starch granules from the respective wash buffer.

d) Purifying the starch that has been freed of proteins

- 5 The starch obtained from Step c), which has been freed from the proteins bound to its surface, is subsequently removed by incubating four times with wash buffer for 10-15 minutes in each case at room temperature under agitation. Each washing step is followed by a centrifugation (5 min, 1,000xg), in order to separate the starch granules from the respective wash buffer. These purification steps serve mainly to remove the
- 10 SDS used in the incubations in Step c).

e) Determination of the concentration of isolated starch

- The amount of starch isolated in Step d) is determined photometrically. After suitable dilution, the optical density of the starch suspension is measured against a calibration
- 15 curve at a wavelength of 600 nm. The linear range of the calibration curve is located between 0 and 0.3 extinction units.

- To produce the calibration curves, starch, for example isolated from leaves of an *Arabidopsis thaliana* *sex1-3* mutant, is dried under vacuum, weighed and absorbed in
- 20 a defined volume of water. The suspension so obtained is diluted with water in several steps in a ratio of 1 to 1 in each case until a suspension of ca. 5 µg starch per ml of water is obtained. The suspensions obtained by the individual dilution steps are measured in the photometer at a wavelength of 600 nm. The absorption values obtained for each suspension are plotted against the concentration of starch in the
- 25 respective suspension. The calibration curve obtained should follow a linear mathematical function in the range from 0 µg starch per ml of water to 0.3 µg starch per ml of water.

f) Storage of isolated starch

The starch can either be used directly without further storage for further tests, or stored in aliquots in 1.5 mL Eppendorf vessels at -20°C. Both the frozen starch and the non-stored, freshly isolated starch can be used, if required, for the methods described in the present invention relating to *in vitro* phosphorylation and/or binding test, for example.

g) Composition of buffers used

1x starch buffer: 20 mM HEPES-KOH, pH 8.0

0.2 mM EDTA

0.5 % Triton X-100

Wash buffer: 50 mM HEPES/KOH, pH 7.2

3. Recombinant expression of an identified starch-phosphorylating protein

- a) Manufacture of a bacterial expression vector containing a cDNA, which codes for a starch-phosphorylating protein

The cDNA coding for a starch-phosphorylating protein can be amplified, for example, using mRNA or poly-A-plus-mRNA from plant tissues as a "template", by means of a polymerase chain reaction (PCR). For this purpose, a reverse transcriptase is first used for the manufacture of a cDNA strand, which is complementary to an mRNA, which codes for a starch-phosphorylating protein, before the cDNA strand concerned is amplified by means of DNA polymerase. So-called "kits" containing substances, enzymes and instructions for carrying out PCR reactions are available for purchase (e.g. SuperScript™ One-Step RT-PCR System, Invitrogen, Prod. No.: 10928-034. The amplified cDNA coding a starch phosphorylating protein can then be cloned in a bacterial expression vector e.g. pDEST™17(Invitrogen). pDEST™17 contains the T7 promoter which is used to initiate the transcription of the T7-RNA polymerase. Furthermore, the expression vector pDEST™17 contains a Shine Dalgarno sequence in the 5'-direction of the T7 promoter followed by a start codon (ATG) and by a so-

called His tag. This His tag consists of six codons directly following one another, which each code for the amino acid histidine and are located in the reading frame of the said start codon. The cloning of a cDNA coding for a starch-phosphorylating protein in pDEST™17 is carried out in such a way that a translational fusion occurs
5 between the codons for the start codon, the His tag and the cDNA coding for a starch-phosphorylating protein. As a result of this, following transcription initiated on the T7 promoter, and subsequent translation, a starch-phosphorylating protein is obtained, which contains additional amino acids containing the His tag on its N-terminus.

10

However, other vectors, which are suitable for expression in microorganisms, can also be used for the expression of a starch-phosphorylating protein. Expression vectors and associated expression strains are known to the person skilled in the art and are also available for purchase from the appropriate dealer in suitable
15 combinations.

b) Manufacture of expression clones in *Escherichia coli*

First of all, an appropriate transformation-competent *E. coli* strain, which chromosomally codes for a T7-RNA polymerase, is transformed with the expression
20 plasmid manufactured under Step a), and subsequently incubated overnight at 30°C on culture medium solidified with agar. Suitable expression strains are, for example, BL21 strains (Invitrogen Prod. No.: C6010-03), which chromosomally code for a T7-RNA polymerase under the control of an IPTG-inducible promoter (lacZ).

25 Bacteria colonies resulting from the transformation can be investigated using methods known to the person skilled in the art to see whether they contain the required expression plasmid containing a cDNA coding for the starch-phosphorylating protein. At the same time, expression clones are obtained.

30 c) Expression of a starch-phosphorylating protein in *Escherichia coli*

First of all, a preliminary culture is produced. To do this, an expression clone obtained in accordance with Step b) is seeded in 30 ml Terrific Broth (TB medium) containing an antibiotic for selection on the presence of the expression plasmid, and incubated overnight at 30°C under agitation (250 rpm).

- 5 A main culture for the expression of a starch-phosphorylating protein is then produced. To do this, in each case, 1 litre Erlenmeyer flasks, each containing 300 ml of TB medium, pre-heated to 30°C, and an antibiotic for selection on the presence of the expression plasmid are each seeded with 10 ml of an appropriate pre-culture and incubated at 30°C under agitation (250 rpm) until an optical density (measured at a
10 wavelength of 600 nm (OD_{600}) of ca. 0.8 is achieved.

- If, for the expression of a starch-phosphorylating protein, an expression plasmid is used, in which the expression of the starch-phosphorylating protein is initiated by means of an inducible system (e.g. the expression vector pDEST™17 in BL21 *E. coli*
15 strains, inducible by means of IPTG), then on reaching an OD_{600} of ca. 0.8, the inductor concerned (e.g. IPTG) is added to the main culture. After adding the inductor, the main culture is incubated at 30°C under agitation (250 rpm) until an OD_{600} of ca. 1.8 is achieved. The main culture is then cooled for 30 minutes on ice before the cells of the main culture are separated from the culture medium by
20 centrifugation (10 minutes at 4,000xg and 4°C).

4. Purification of a starch-phosphorylating protein

a) Breaking down of cells expressing a starch-phosphorylating protein

- The cells obtained in Step c), Item 3 General Methods are resuspended in lysis
25 buffer. In doing so, ca. 4 ml lysis buffer is added to about 1 g of cells. The resuspended cells are then incubated for 30 minutes on ice before they are broken down using an ultrasonic probe (Baudelin Sonoplus UW 2070, Baudelin electronic, Berlin, settings: Cycle 6, 70%, 1 minute) under continuous cooling by means of the ice. Care must be taken here to ensure that the cell suspension is not heated too
30 much during the ultrasonic treatment. The suspension obtained after the ultrasonic

treatment is centrifuged (12 minutes at 20,000xg, 4°C) and the supernatant obtained after centrifugation is filtered using a filter with a pore size of 45 µm.

b) Purification of the starch-phosphorylating protein

- 5 If the starch-phosphorylating protein expressed in *E. coli* cells is a fusion protein with a His tag, then purification can take place using nickel ions, to which the His tag binds with greater affinity. To do this, 25 ml of the filtrate obtained in Step d) is mixed with 1 ml Ni-agarose slurry (Qiagen, Prod. No.: 30210) and incubated for 1 hour on ice. The mixture of Ni-agarose slurry and filtrate is subsequently spread over a
- 10 polystyrene column (Pierce, Prod. No.: 29920). The product, which runs through the column, is discarded. The column is next washed by adding 8 ml of lysis buffer, the product, which runs through the column, again being discarded. Elution of the starch-phosphorylating protein then takes place by fractionated addition to the column of 1 ml E1 buffer twice, followed by 1 ml E2 buffer once and subsequently 1 ml E3 buffer
- 15 five times. The product, which runs through the column, which is produced by adding the individual fraction of the appropriate elution buffer (E1, E2, E3 buffer) to the column, is collected in separate fractions. Aliquots of these fractions are subsequently analysed by means of denaturing SDS acrylamide gel electrophoresis followed by Coomassie Blue staining. The fractions, which contain the starch-
- 20 phosphorylating protein in sufficient quantity and satisfactory purity, are purified and concentrated using pressurised filtration at 4°C. Pressurised filtration can be carried out, for example, using an Amicon cell (Amicon Ultrafiltration Cell, Model 8010, Prod. No.: 5121) using a Diaflo PM30 membrane (Millipore, Prod. No.: 13212) at 4°C. Other methods known to the person skilled in the art can also be used for
- 25 concentration however.

c) Composition of buffers used

Lysis buffer:	50 mM	HEPES	
		300 mM	NaCl
		10 mM	Imidazole

- 57 -

pH 8.0 (adjust with NaOH)

1 mg/ml Lysozyme (add immediately before
using the buffer)

5 ¼ tablet per 10 ml of protease inhibitors Complete EDTA free, (Roche Product No.:
1873580) (add immediately before using the buffer)

10 Elution buffer E1: 50 mM HEPES
300 mM NaCl
50 mM Imidazole
pH 8.0 (adjust with NaOH)

15 Elution buffer E2: 50 mM HEPES
300 mM NaCl
75 mM Imidazole
pH 8.0 (adjust with NaOH)

20 Elution buffer E3: 50 mM HEPES
300 mM NaCl
250 mM Imidazole
pH 8.0 (adjust with NaOH)

5. Recombinant expression of an R1 protein

The recombinant expression of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the

recombinant expression of a starch-phosphorylating protein described above under Item 3, General Methods.

6. Purification of an R1 protein

- 5 The purification of an R1 protein is described in the literature (Ritte et al., 2002, PNAS 99, 7166-7171; Mikkelsen et al., Mikkelsen et al., 2004, Biochemical Journal 377, 525-532), but can also be carried out in accordance with the methods relating to the purification of a starch-phosphorylating protein described above under Item 4, General Methods if an R1 fusion protein, which contains a His tag, is produced by
10 expression of R1 in *E. coli* cells.

7. In vitro manufacture of phosphorylated starch starting from non-phosphorylated starch

a) *In vitro* phosphorylation of non-phosphorylated starch

- 15 Starch, which does not contain starch phosphate (e.g. isolated from leaves of *Arabidopsis thaliana* *sex1-3* mutants using the methods described above under Item 2, General Methods), is mixed with R1 buffer and with purified R1 protein (ca. 0.25 µg R1 protein per mg starch) in order to produce a starch content of 25 mg per ml. This reaction preparation is incubated overnight (ca. 15 h) at room temperature under
20 agitation. R1 bound to the starch present in the reaction preparation is removed on completion of the reaction by washing four times with ca. 800 µl 0.5 % SDS in each case. Subsequently, the SDS still present in the *in vitro* phosphorylated starch is removed by washing five times with 1 ml wash buffer in each case. All washing steps are carried out at room temperature for 10 to 15 minutes under agitation. Each
25 washing step is followed by a centrifugation (2 min, 10,000xg), in order to separate the starch granules from the respective SDS buffer or wash buffer.

b) Composition of buffers used

R1 buffer: 50 mM HEPES/KOH, pH 7,5
1 mM EDTA
6 mM MgCl₂
0.5 mM ATP

5

Wash buffer: 50 mM HEPES/KOH, pH 7.2

8. Binding of proteins to phosphorylated starch or non-phosphorylated starch

- 10 a) Isolation of P-starch protein complexes or non-phosphorylated starch protein complexes

Ca. 50 mg of P-starch or ca. 50 mg of non-phosphorylated starch are resuspended in separate preparations in ca. 800 µl of protein extract in each case. The protein concentration of the protein extracts should be ca. 4 mg to 5 mg per ml in each case.

- 15 The incubation of the P-starch or non-phosphorylated starch with protein extracts is carried out at room temperature for 15 minutes at 4°C under agitation. On completion of the incubation, the reaction preparations are centrifuged out using a Percoll cushion (4 ml) (15 minutes, 3500 rpm, 4°C). After centrifugation, proteins that are not bound to phosphorylated starch or P-starch will be found in the supernatant and can
- 20 be removed with a Pasteur pipette. The supernatant is discarded. The sedimented pellet containing P-starch and non-phosphorylated starch, including the proteins bound to the respective starches (P-starch protein complexes or non-phosphorylated starch protein complexes respectively), obtained after centrifugation is washed twice with 1 ml of wash buffer in each case (see above, General Methods under item 7.b)
- 25 by incubating for 3 minutes at 4°C in each case under agitation. The washing step is followed by a centrifugation (5 minutes, 8000 rpm, 4°C in a table centrifuge, Hettich EBA 12R) in order to separate the P-starch or non-phosphorylated starch respectively from the wash buffer.

c) Composition of buffers used

20

Percoll: Percoll is dialysed overnight against a solution consisting of and 25 mM HEPES / KOH, pH 7.0

25 **9. Separation of proteins, which bind to P-starch and/or non-phosphorylated starch**

The dissolved proteins obtained in Step c) under Item 8. General Methods relating to the binding of proteins to P-starch or non-phosphorylated starch respectively are incubated for 5 minutes at 95°C in each case and subsequently separated using

denaturing polyacrylamide gel electrophoresis. In doing so, an equal volume is applied to the acrylamide gel in each case for the dissolved proteins obtained by binding to P-starch and for those obtained by binding to non-phosphorylated starch. The gel obtained on completion of electrophoresis is stained at least overnight with colloidal Coomassie (Roth, Karlsruhe, Roti-Blue Rod. No.: A152.1) and subsequently decolourised in 30 % methanol, 5 % acetic acid, or in 25% methanol.

10. Identification and isolation of proteins, which bind to P-starch and/or non-phosphorylated starch

- 10 a) Identification of proteins with increased binding activity towards P-starch in comparison with non-phosphorylated starch

Proteins, which, after separation by means of acrylamide gel electrophoresis and subsequent visualisation by staining (see above, Item 9, General Methods), exhibit an increased signal after binding to P-starch in comparison with a corresponding signal after binding to non-phosphorylated starch, have increased bonding activity towards P-starch in comparison with non-phosphorylated starch. By this means, it is possible to identify proteins, which have increased binding activity towards P-starch in comparison with non-phosphorylated starch. Proteins, which have increased binding activity towards P-starch in comparison with non-phosphorylated starch, are excised from the acrylamide gel.

- b) Identification of proteins, which have increased binding activity towards P-starch in comparison with non-phosphorylated starch

Proteins identified in accordance with Step a) are digested with trypsin and the peptides obtained are analysed by means of MALDI-TOF to determine the masses of the peptides obtained. Trypsin is a sequence-specific protease, i.e. trypsin only splits proteins at a specified position when the proteins concerned contain certain amino acid sequences. Trypsin always splits peptide bonds when the amino acids arginine and lysine follow one another starting from the N-terminus. In this way, it is possible to theoretically determine all peptides that would be produced following the trypsin

digestion of an amino acid sequence. From the knowledge of the amino acids coding for the theoretically determined peptides, the masses of the peptides, which are obtained after theoretical trypsin digestion, can also be determined. Databases (e.g. NCBI Inr <http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm>; Swissprot <http://cbrg.inf.ethz.ch/Server/MassSearch.html>), which contain information concerning the masses of peptides after theoretical trypsin digestion, can therefore be compared with the real masses of peptides of unknown proteins obtained with MALDI-TOF-MS. Amino acid sequences, which have the same peptide masses after theoretical and/or real trypsin digestion, are to be looked upon as being identical. The databases concerned contain both peptide masses of proteins, the function of which has already been shown, and also peptide masses of proteins, which up to now only exist hypothetically by derivation from amino acid sequences starting from nucleic acid sequences obtained in sequencing projects. The actual existence and the function of such hypothetical proteins has therefore seldom been shown and, if there is a function at all, then this is usually based only on predictions and not on an actual demonstration of the function.

Bands containing proteins obtained in accordance with Step a) are excised from the acrylamide gel; the excised acrylamide piece is reduced and decolourised by incubating for approximately half an hour at 37°C in ca. 1 ml 60% 50mM NH_4HCO_3 , 40% acetonitrile. The decolourising solution is subsequently removed and the remaining gel dried under vacuum (e.g. Speedvac). After drying, trypsin solution is added to digest the proteins contained in the gel piece concerned. Digestion takes place overnight at 37°C. After digestion, a little acetonitrile is added (until the acrylamide gel is stained white) and the preparation dried under vacuum (e.g. Speedvac). When drying is complete, just enough 5% formic acid is added to cover the dried constituents and incubated for a few minutes at 37°C. The acetonitrile treatment followed by drying is repeated once more. The dried constituents are subsequently absorbed in 0.1% TFA (trifluoroacetic acid, 5 μl to 10 μl) and dripped onto a carrier in ca. 0.5 μl portions. Equal amounts of matrix (ϵ -Cyano-4-hydroxycinnamic acid) are also applied to the carrier. After crystallising out the matrix, the masses of peptides are determined by means of MALDI-TOF-MS-MS (e.g. Burkner

Reflex™ II, Bruker Daltonic, Bremen) With the masses obtained, databases are searched for amino acid sequences, which give the same masses after theoretical trypsin digestion. In this way, amino acid sequences can be identified, which code for proteins, which preferably bind to phosphorylated alpha-1,4-glucans and/or which
5 need P-alpha-1,4-glucans as a substrate.

11. Method for demonstrating starch-phosphorylating activity of a protein

a) Incubation of proteins with P-starch and/or non-phosphorylated starch

In order to demonstrate whether a protein has starch-phosphorylating activity, proteins to be investigated can be incubated with starch and radioactively labeled
10 ATP. To do this, ca. 5 mg of P-starch or ca. 5 mg of non-phosphorylated starch are incubated with the protein to be investigated (0.01 µg to 5.0 µg per mg of starch used) in 500 µl phosphorylation buffer for 10 minutes to 30 minutes at room temperature under agitation. The reaction is subsequently stopped by the addition of
15 SDS up to a concentration of 2% (weight/volume). The starch granules in the respective reaction mixture are centrifuged out (1 minute, 13,000xg), and washed once with 900 µl of a 2 % SDS solution and four times each with 900 µl of a 2 mM ATP solution. Each washing step is carried out for 15 minutes at room temperature under agitation. After each washing step, the starch granules are separated from the
20 respective wash buffer by centrifugation (1 min, 13,000xg).

In addition, when carrying out an experiment to demonstrate starch-phosphorylating activity of a protein, further reaction preparations, which do not contain protein or contain inactivated protein, but which are otherwise treated in the same way as the reaction preparations described, should be processed as so-called controls.

25

b) Determination of the amount of phosphate residues incorporated in the P-starch and/or non-phosphorylated starch due to enzymatic activity

The starch granules obtained in accordance with Step a) can be investigated for the presence of radioactively labeled phosphate residues. To do this, the respective
30 starch is resuspended in 100 µl of water and mixed with 3 ml of scintillation cocktail in

each case (e.g. Ready Safe™, BECKMANN Coulter) and subsequently analysed using a scintillation counter (e.g. LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTER™).

5 c) Identification of proteins, which preferably use P-starch as a substrate

If a protein is incubated in separate preparations, once with P-starch and once with non-phosphorylated starch, in accordance with the method described under a), then, by comparing the values for the presence of starch phosphate obtained according to Step b), it can be determined whether the protein concerned has incorporated more
 10 phosphate in P-starch in comparison with non-phosphorylated starch. Thus, proteins which can introduce phosphate into P-starch but not into non-phosphorylated starch can also be identified, i.e., proteins which already require phosphorylated starch as substrate for a further phosphorylation reaction can be identified.

15 d) Composition of buffers used

Phosphorylation buffer:	50 mM	HEPES/KOH, pH 7.5
	1 mM	EDTA
	6 mM	MgCl ₂
	0.01 to 0.5 mM	ATP

20 0.2 to 2 µCi per ml randomised ³³P-ATP (alternatively, ATP, which contains a phosphate residue, which is specifically labeled in the gamma position, can also be used)

In conjunction with the present invention, the term "randomised ATP" is to be
 25 understood to mean ATP, which contains labeled phosphate residues both in the gamma position and in the beta position (Ritte et al. 2002, PNAS 99, 7166-7171). Randomised ATP is also described in the scientific literature as beta/gamma ATP. A method for manufacturing randomised ATP is described in the following.

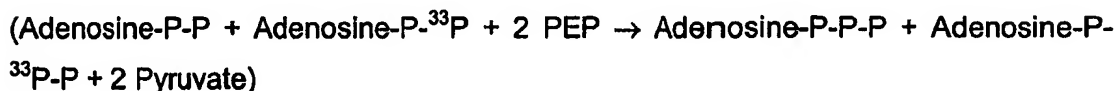
i) Manufacture of randomised ATP

The method described here for manufacturing randomised ATP using enzyme catalysed reactions is based on the following reaction mechanisms:

1. reaction step 1



2. reaction step 2



- 10 The reaction equilibria lie on the product side but, in spite of this, this reaction produces a mixture consisting mainly of $\beta^{33}\text{P-ATP}$ and some $\gamma^{33}\text{P-ATP}$.

ii) Carrying out the 1st reaction step

- ATP (100 μCi , 3000 Ci per mmol), which contains a phosphate residue labeled with ^{33}P in the gamma position (Hartmann Analytic, 10 $\mu\text{Ci}/\mu\text{l}$), is incubated with 2 μl myokinase (AMP-phosphotransferase, from rabbit muscle; SIGMA, Prod. No.: M3003 3.8 mg/ml, 1,626 units/mg) in 90 μl randomising buffer for 1 hour at 37°C. The reaction is subsequently stopped by incubating for 12 minutes at 95°C before the reaction preparation is purified by means of centrifugal filtration using a Microcon YM 20 10 filter (Amicon, Millipore Prod. No. 42407) at 14,000xg for at least 10 minutes.

iii) Carrying out the 2nd reaction step

- 2 μl pyruvate kinase (see below for how to manufacture an appropriate solution) and 3 μl 50 mM PEP (phosphoenolpyruvate) are added to the filtrate obtained in Step ii). 25 This reaction mixture is incubated for 45 minutes at 30°C before the reaction is stopped by incubating at 95°C for 12 minutes. The reaction mixture is subsequently centrifuged (2 minutes, 12,000 rpm in an Eppendorf table centrifuge). The

supernatant containing randomised ATP obtained after centrifugation is removed, aliquoted and can be stored at -20°C.

Manufacture of the pyruvate kinase solution

- 5 15 µl pyruvate kinase (from rabbit muscle, Roche, Prod. No. 12815, 10 mg/ml, 200 units/mg at 25 °C) are centrifuged out, the supernatant discarded and the pellet absorbed in 27 µl pyruvate kinase buffer.

iv) Buffers used

10	Pyruvate kinase buffer:	50 mM	HEPES/KOH pH 7.5
		1 mM	EDTA
15	Randomising buffer:	100 mM	HEPES/KOH pH 7.5
		1 mM	EDTA
		10 %	Glycerol
		5 mM	MgCl ₂
		5 mM	KCl
		0.1 mM	ATP
		0.3 mM	AMP

20 12. Demonstration of the autophosphorylation of a protein

In order to demonstrate whether a protein has autophosphorylating activity, proteins to be investigated can be incubated with radioactively labeled ATP. To do this, proteins to be investigated (50 µg to 100 µg) are incubated in 220 µl phosphorylation buffer (see above, Item 12 d), General Methods) for 30 minutes to 90 minutes at
 25 room temperature under agitation. The reaction is then stopped by adding EDTA up to a final concentration of 0.11 M. Ca. 2 µg to 4 µg of protein is then separated using denaturing polyacrylamide electrophoresis (7.5% acrylamide gel). The gel obtained

after polyacrylamide gel electrophoresis is subjected to autoradiography. Proteins, which exhibit a signal in the autoradiography, carry a radioactive phosphate residue.

**13. Identification of the C-atom positions of the glucose molecules of an
5 alpha-1,4-glucan, into which phosphate residues are introduced by a
 starch-phosphorylating protein**

Which C-atom positions of the glucose molecules of an alpha-1,4-glucan are phosphorylated by a protein can be demonstrated in a controlled manner by hydrolysis of the phosphorylated glucans obtained by means of an appropriate
10 protein *in vitro*, subsequent separation of the glucose monomers obtained after hydrolysis, followed by measurement of the phosphate incorporated by an appropriate protein in certain fractions of the glucose molecules.

a) Total hydrolysis of the alpha-1,4-glucans

15 Water suspensions containing alpha-1,4-glucan are centrifuged, the sedimented pellet subsequently resuspended in 0.7 M HCl (Baker, for analysis) and incubated for 2 hours at 95°C under agitation. On completion of incubation, the samples are briefly cooled and centrifuged (e.g. 2 minutes 10,000xg). The supernatant obtained is transferred to a new reaction vessel and neutralised by the addition of 2 M NaOH
20 (Baker, for analysis). If a pellet remains, it is resuspended in 100 µl of water and the quantity of labeled phosphate present therein is determined as a control.

The neutralised supernatant is subsequently centrifuged over a 10 kDa filter. By measuring an aliquot of the filtrate obtained, the quantity of labeled phosphate in the
25 filtrate is determined using a scintillation counter, for example.

**b) Fractionation of the hydrolysis products and determination of the
phosphorylated C-atom positions**

The neutralised filtrates of the hydrolysis products obtained by means of Step a) can be separated (when using radioactively labeled ATP about 3000 cpm) using high-pressure anion exchange chromatography (HPAE), for example. The neutralised filtrate can be diluted with H₂O to obtain the volume required for HPAE. In addition, glucose-6-phosphate (ca. 0.15 mM) and glucose-3-phosphate (ca. 0.3 mM) are added to the appropriate filtrates in each case as an internal control. Separation by means of HPAE can be carried out, for example, using a Dionex DX 600 Bio Lc system using a CarboPac PA 100 column (with appropriate pre-column) and a pulsed amperometric detector (ED 50). In doing so, before injecting the sample, the column is first rinsed for 10 minutes with 99% eluent C and 1% eluent D. A sample volume of 60 µl is then injected. The elution of the sample takes place under the following conditions:

Flow rate: 1 ml per minute

Gradient: linearly increasing from 0 minutes to 30 minutes

	Eluent C	Eluent D
0 minutes	99%	1%
30 minutes	0%	100%
35 Minutes	0%	100%
Run terminated		

20

The hydrolysis products eluted from the column are collected in individual fractions of 1 ml each. As, in each case, non-labeled glucose-3-phosphate (Ritte et al. 2002, PNAS 99, 7166-7171) and non-labeled glucose-6-phosphate (Sigma, Prod. No.: G7879) have been added to the injected samples of hydrolysis products as internal standards, the fractions, which contain either glucose-3-phosphate or glucose-6-phosphate, can be determined by means of pulsed amperometric detection. By measuring the amount of labeled phosphates in the individual fractions and subsequently comparing with the fractions, which contain glucose-3-phosphate or glucose-6-phosphate, this can be used to determine those fractions, containing labeled glucose-6-phosphate or labeled glucose-3-phosphate. The amount of labeled

phosphate in the fraction concerned is determined. From the ratios of the amounts of glucose-3-phosphate to glucose-6-phosphate measured for labeled phosphate in the individual hydrolysis products, it can now be determined which C-atom position is preferably phosphorylated by an alpha-1,4-glucan phosphorylating enzyme.

5

c) Buffers used

Eluent C: 100 mM NaOH

Eluent D: 100 mM NaOH

500 mM sodium acetate

10

14. Preparation of the samples for the sequencing using Q-TOF-MS-MS

a) General remarks

Isolated proteins which can also be present in the form of bands excised from polyacrylamide gels, are first cleaved into smaller fragments by means of a trypsin digestion. The peptides formed are introduced into a hybrid mass spectrometer in which a time-of-flight (TOF) mass spectrometer is coupled to a quadrupole mass spectrometer. In the first phase of the measurement the first mass spectrometer (the quadrupole) is "switched off" and the masses of the peptides formed in the digestion can be determined in the TOF mass spectrometer. In the second phase a selected peptide is "filtered out" in the quadrupole, i.e., only this peptide can pass the quadrupole, all the others are deflected. The peptide is then broken by colliding with charged gas molecules in the "collision cell". In this case the "breaks" occur mainly at the peptide bonds. As a result, more or less statistically distributed peptide fragments which differ in mass are formed. The amino acid sequence of the peptides can then be determined by "sorting" these fragments. If overlapping peptides are obtained, the amino acid sequence of a protein can thus be obtained. The use of mass spectroscopy for identification and sequencing is known to the person skilled in the art and is sufficiently described in the specialist literature [e.g. P. Michael Conn (Ed.)];

2003, Humana Press, New Jersey, ISBN: 1-58829-340-8]; J.R. Chapman (Ed.), 2000, Humana Press, SBN: 089603609X].

b) Reduction and alkylation of cysteine residues of proteins

- 5 The cysteine residues containing the amino acid sequences of the proteins to be analysed can be reduced/alkylated by means of gel electrophoresis before separation of the proteins. For this purpose, the proteins which are to be separated by means of gel electrophoresis are mixed with SDS sample buffer (must not contain any DTT or beta-mercaptoethanol). Freshly prepared DTT is then added to these
- 10 samples up to a final concentration of 10 mM and the sample incubated for 3 minutes at 95°C. After cooling the sample to room temperature, freshly prepared iodoacetamide is added up to a final concentration of 20 mM. The sample is incubated for 20 minutes at room temperature in the dark. The proteins present in the samples are then separated by means of acrylamide gel electrophoresis.

15

c) Isolation of the proteins from the acrylamide gel

- Protein bands containing proteins whose sequences are to be determined are excised using a clean scalpel as "edgeless" as possible and reduced (ca. 1 mm³-cube). The reduced gel pieces are placed in a 0.5 ml or 1.5 ml reaction vessel and
- 20 sedimented by short centrifugation.

d) Decolourisation of the excised gel pieces

- If gels stained using silver ions were used, the gel pieces obtained according to step c) are completely covered with a solution containing 30 mM K-ferricyanide and 100
- 25 mM Na-thiosulphate in the ratio 1:1 and agitated (Vortex) until the gel pieces are completely decolourised. The decolourising solution is then removed and the gel pieces are washed three times with 200 µl of high-purity water in each case (conductivity ca. 18 MOhm).

If gels stained with Coomassie Blue were used, the gel pieces obtained according to step c) are incubated with a solution containing high-purity water and acetonitrile (degree of purity: at least HPLC pure) in the ratio 1:1 twice for 15 minutes in each case under agitation. The volume of the decolourising solution should correspond to
5 ca. twice the volume of the gel. The washing solution is removed after each washing step.

After decolourisation has been completed, the gel pieces are mixed with one volume (relative to the gel pieces) of acetonitrile and incubated for 15 minutes at room
10 temperature under agitation. The acetonitrile is removed and the gel pieces mixed with one volume of 100 mM ammonium bicarbonate, mixed and incubated for 5 minutes at room temperature. Acetonitrile is then added so as to give a ratio of 1:1 relative to the quantity of ammonium bicarbonate and acetonitrile. Incubation is carried out for a further 15 minutes at room temperature before the solution is
15 removed and the remaining gel pieces are dried under vacuum (e.g. Speedvac).

e) Trypsin digestion of the proteins in the gel pieces

Trypsin solution (10 ng of trypsin per μ l of 50 mM ammonium bicarbonate) is added in 10 μ l portions to the dry gel pieces obtained according to step d). After every
20 addition of trypsin solution, incubation on ice is carried out for 10 minutes in each case. Trypsin solution is added in portions until the gel pieces do not swell any further and are completely covered by trypsin solution. The trypsin solution is then removed and the gel pieces are incubated overnight at 37°C.

25 f) Isolation of the peptides from the acrylamide gel

The samples obtained according to step e) are briefly centrifuged in order to collect the liquid contained in the reaction vessel, the liquid is removed and transferred to a new reaction vessel. The gel pieces are treated for 2 minutes with ultrasound (ultrasound water bath). The remaining gel pieces are then mixed with once their
30 volume of 25 mM ammonium bicarbonate solution and incubated for 20 minutes

under agitation. Acetonitrile is then added so that a ratio of ammonium bicarbonate to acetonitrile of 1:1 is adjusted and incubation is carried out at room temperature for a further 15 minutes under agitation. After incubation has been completed, the samples are treated with ultrasound again for 2 minutes before the liquid is removed and
5 combined with the liquid which had been removed previously. The remaining gel pieces are mixed with once their volume of a solution containing 5% formic acid and acetonitrile in the ratio 1:1 and incubated for 15 minutes at room temperature under agitation. The liquid is removed and combined with the liquid which had been removed previously. The incubation of the gel pieces in 5% formic acid / acetonitrile
10 (ratio 1:1) is repeated and the liquid obtained is likewise added to the previously collected liquids. The combined supernatants contain the peptides to be sequenced and are concentrated to ca. 15 µl in the vacuum centrifuge (Speedvac) at 60°C. The peptides thus obtained can be stored at 20°C until they are analysed using Q-TOF. Before the proteins can be sequenced in the mass analysis, they can be desalted
15 using methods known to the person skilled in the art.

15. Transformation of rice plants

Rice plants were transformed in accordance with the methods described by Hiei et al. (1994, Plant Journal 6(2), 271-282).

20

16. Transformation of potato plants

Potato plants were transformed using agrobacterium as described by Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29).

25 17. Determination of the starch phosphate content

Determination of the C-6 phosphate content

In the starch the positions C2, C3 and C6 of the glucose units can be phosphorylated. 50 mg of starch was hydrolysed in 500 µl of 0.7 M HCl for 4 h at

95°C to determine the C6-P content of the starch. The preparations were then centrifuged for 10 min at 15,500 g and the supernatants removed. From the supernatants 7 µl is mixed with 193 µl of imidazole buffer (100 mM imidazole, pH 7.4; 5 mM MgCl₂, 1 mM EDTA and 0.4 mM NAD). The measurement was made using a
5 photometer at 340 nm. After a base absorption had been established, the enzyme reaction was started by adding 2 units of glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, Boehringer Mannheim). The change in absorption is directly proportional to the concentration of the G-6-P content of the starch.

10 b) Determination of the total phosphate content

The total phosphate content was determined using the Ames method (Methods in Enzymology VIII, (1966), 115-118).

Approximately 50 mg of starch is mixed with 30 µl of ethanol magnesium nitrate solution and ashed for three hours at 500°C in a muffle furnace. The residue is mixed
15 with 300 µl of 0.5 M hydrochloric acid and incubated for 30 min at 60°C. An aliquot is then made up to 300 µl of 0.5 M hydrochloric acid, added to a mixture of 100 µl of 10% ascorbic acid and 600 µl of 0.42% ammonium molybdate in 2 M sulphuric acid and incubated for 20 min at 45°C.

20 c) Determination of the content of C-6 phosphate and C-3 phosphate

To determine the content of phosphate bound in the C-6 position and in the C-3 position of the glucose molecules of an alpha-1,4-glucan, the glucans concerned can be separated using HPAE after total hydrolysis using the method specified under General Methods Item 13. The quantities of glucose-6-phosphate and glucose-3-
25 phosphate can be determined by integrating the individual peak areas obtained after HPEA separation. The quantity of glucose-6-phosphate and glucose-3-phosphate in the samples to be studied can be determined by comparing the peak area for glucose-6-phosphate and glucose-3-phosphate obtained in the unknown samples, with the peak areas obtained after separation using HPAE with known quantities of
30 glucose-6-phosphate and glucose-3-phosphate.

Examples

1. Isolation of a protein from *Arabidopsis thaliana*, which has increased binding activity with respect to P-starch in comparison with non-phosphorylated starch

a) Manufacture of protein extracts from *Arabidopsis thaliana*

Protein extracts were manufactured from approximately 7 g of leaves (fresh weight) of *Arabidopsis thaliana* (Ökotyp Columbia, Col-O) in accordance with the method described under Item 1, General Methods.

b) Isolation of starch granules from leaves of *sex1-3* mutants of *Arabidopsis thaliana*

Starch granules were isolated from approximately 20 g (fresh weight) of leaves of a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with the method described under Item 2, General Methods.

c) *In vitro* phosphorylation of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* with purified R1 protein.

About 30 mg of non-phosphorylated starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* was phosphorylated in accordance with the method described under Item 7, General Methods, by means of an R1 protein recombinantly expressed in *E. coli* and purified. The methods described in Ritte et al. (2002, PNAS 99, 7166-7171) were used for the expression of the R1 protein in *E. coli* and for the subsequent purification.

d) Isolation of proteins, which bind to P-starch and/or non-phosphorylated starch

Protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed in a Preparation A with 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) using the method described under Item 8 a), General Methods.

5

In a second Preparation B, protein extracts of *Arabidopsis thaliana*, obtained in accordance with Step a), were incubated and washed with 50 mg of the non-phosphorylated starch manufactured in accordance with Step b) using the method described under Item 8 a), General Methods.

10

Subsequently, the proteins bound to the P-starch of Preparation A and to the non-phosphorylated starch of Preparation B were dissolved in accordance with the method described under Item 8 b), General Methods.

15 In a third Preparation C, 50 mg of the *in vitro* phosphorylated starch manufactured in accordance with Step c) were incubated and washed using the method described under Item 8 a), General Methods. Preparation C contained no protein extracts however.

20 e) Separation of the proteins obtained in accordance with Step d) by means of acrylamide gel electrophoresis

The proteins of Preparations A, B and C obtained in Step d) were separated by means of a 9% acrylamide gel under denaturing conditions (SDS) using the method described under Item 9, General Methods, and subsequently stained with Coomassie

25 Blue. The stained gel is shown in Fig. 1. It can be clearly seen that a protein, which has a molecular weight of ca. 130 kDa in denaturing acrylamide gel referred to a protein standard marker (Trace M), preferably binds to phosphorylated starch (Trace P) in comparison with non-phosphorylated starch (K).

f) Identification of the protein, which preferably binds to P-starch in comparison with non-phosphorylated starch

The band of the protein with a molecular weight of ca. 130 kDa identified in Step e) was excised from the gel. The protein was subsequently released from the acrylamide as described under General Methods 10 b), digested with trypsin and the peptide masses obtained determined by means of MALD-TOF-MS. The so-called "fingerprint" obtained by MALDI-TOF-MS was compared with fingerprints of theoretically digested amino acid molecules in databases (Mascot: http://www.matrixscience.com/search_form_select.html; ProFound: http://129.85.19.192/profound_bin/WebProFound.exe; PepSea: <http://195.41.108.38/PepSeaIntro.html>). As such a fingerprint is very specific to a protein, it was possible to identify an amino acid molecule. Using the sequence of this amino acid molecule, it was possible to isolate a nucleic acid sequence from *Arabidopsis thaliana* coding for an OK1 protein. The protein identified using this method was designated as A.t.-OK1. After analysing the amino acid sequence from *Arabidopsis thaliana*, it was found that this deviates from the sequence present in the database (NP 198009, NCBI). The amino acid sequence shown in SEQ ID No 2 codes for the A.t.-OK1 protein. SEQ ID No 2 contains deviations when compared with the sequence in the database (Acc.: NP 198009.1, NCBI). The amino acids 519 to 523 (WRLCE) and 762 to 766 (VRARQ) contained in SEQ ID No 2 are not in the sequence, which is present in the database (ACC.: NP 198009.1). NP 198009.1). Compared to version 2 of the database sequence (Acc.: NP 198009.2) the amino acid sequence shown in SEQ ID NO 2 contains the additional amino acids 519 to 523 (WRLCE).

25 2. Cloning of a cDNA, which codes for the Identified OK1 protein

The A.t.-OK1 cDNA was isolated using reverse PCR using mRNA isolated from leaves of *Arabidopsis thaliana*. To do this, a cDNA Strand was synthesised by means of reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen Prod. No.: 11904-018), which was then amplified using DNA polymerase (Expand High Fidelity PCR Systems, Roche Prod. No.: 1732641). The amplified product obtained from this PCR reaction was cloned into the vector pGEM®(-T

(Invitrogen Prod. No.: A3600). The plasmid obtained is designated A.t.-OK1-pGEM®-T, the cDNA sequence coding for the A.t.-OK1 protein was determined and is shown under SEQ ID NO. 1.®

- 5 The sequence shown under SEQ ID NO 1 is not the same as the sequence, which is contained in the database. This has already been discussed for the amino acid sequence coding for an A.t.-OK1 protein.

Conditions used for the amplification of the cDNA coding for the A.t.-OK1 protein

10 First strand synthesis:

The conditions and buffer specified by the manufacturer were used. In addition, the reaction preparation for the first strand synthesis contained the following substances:

3 µg Total RNA

5 µM 3'-primer (OK1rev1:5'-GACTCAACCACATAACACACAAAGATC)

15 0.83 µM dNTP Mix

The reaction preparation was incubated for 5 minutes at 75°C and subsequently cooled to room temperature.

- The 1st strand buffer, RNase inhibitor and DTT were then added and incubated for 2 minutes at 42°C before 1 µL Superscript RT DNA polymerase was added and the
20 reaction preparation incubated for 50 minutes at 42°C.

Conditions for the amplification of the first strand by means of PCR:

1 µL of the reaction preparation of the first strand synthesis

0.25 µM 3'Primer (OK1rev2:5'- TGGTAACGAGGCAAATGCAGA)

0.25 µM 5'Primer (OK1fwd2:5'-

25 ATCTCTTATCACACCACCTCCAATG)

Reaction conditions:

Step 1 95°C 2 min

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	Step 2	94°C 20 sec
	Step 3	62°C 30 sec
	Step 4	68°C 4 minutes
	Step 5	94°C 20 sec
5	Step 6	56°C 30 sec
	Step 7	68°C 4 minutes
	Step 8	68°C 10 minutes

The reaction was first carried out in accordance with Steps 1 to 4. Ten repeats (cycles) were carried out between Step 4 and Step 2, the temperature of Step 3 being reduced by 0.67°C after each cycle. This was subsequently followed by the reaction in accordance with the conditions specified in Steps 5 to 8. Twenty five repeats (cycles) were carried out between Step 7 and Step 5, the time of Step 7 being increased by 5 sec on each cycle. On completion of the reaction, the reaction was cooled to 4°C.

15 3. Manufacture of a vector for the recombinant expression of cDNA of the OK1 protein

Following amplification by means of PCR by using the plasmid A.t.-OK1-pGEM® as a template using Gateway Technology (Invitrogen), the sequence coding the OK1 protein from *Arabidopsis thaliana* was first cloned in the vector pDONOR™ 201 (Invitrogen Prod. No.: 11798-014). Subsequently, the coding region of the OK1 protein from the vector obtained was cloned by sequence-specific recombination into the expression vector pDEST™17 (Invitrogen Prod. No.: 11803-014). The expression vector obtained is designated as A.t.-OK1-pDEST™1. The cloning resulted in a translational fusion of the cDNA coding for the A.t.-OK1 protein with the nucleotides present in the expression vector pDEST™17. The nucleotides originating from the vector pDEST™17, which are translationally fused with the cDNA coding the A.t.-OK1 protein, code for 21 amino acids. These 21 amino acids include, amongst others, the start codon (ATG) and a so-called His tag (6 histidine residues directly after one another). After translation of these translationally fused sequences, this

results in an A.t.-OK1 protein, which has the additional 21 amino acids coded for by nucleotides originating from the vector at its N-terminus. The recombinant A.t.-OK1 protein resulting from this vector therefore contains 21 additional amino acids originating from the vector pDEST™17 at its N-terminus.

5

4. Heterologous expression of the OK1 protein in *E. coli*

The expression vector A.t.-OK1-pDEST™17 obtained in accordance with Example 3 was transformed in the *E. coli* strain BL21 Star™ (DE3) (Invitrogen, Prod. No. C6010-03). A description of this expression system has already been given above (see Item 10 3, General Methods). Bacteria clones, containing the vector A.t.-OK1-pDEST™17, resulting from the transformation were first used to manufacture a preliminary culture, which was subsequently used for inoculating a main culture (see Item 3.c, General methods). The preliminary culture and the main culture were each incubated at 30°C under agitation (250 rpm). When the main culture had reached an OD₆₀₀ of ca. 0.8, 15 the expression of the recombinant A.t.-OK1 protein was induced by the addition of IPTG (isopropyl-beta-D-thiogalactopyranoside) until a final concentration of 1 mM was achieved. After the addition of IPTG, the main culture was incubated at 30°C under agitation (250 rpm) until an OD₆₀₀ of ca. 1.8 was achieved. The main culture was then cooled for 30 minutes on ice before the cells of the main culture were 20 separated from the culture medium by centrifugation (10 minutes at 4,000xg and 4°C).

5. Purification of the recombinantly expressed OK1 protein

The purification and concentration of the A.t.-OK1 protein from cells obtained in 25 accordance with Example 4 was carried out using the method described under Item 4, General Methods.

6. Demonstration of starch-phosphorylating activity of the OK1 protein

The starch-phosphorylating activity of the A.t.-OK1 protein was demonstrated in accordance with the method described under Item 11, General Methods. In doing so, 5 µg of purified A.t.-OK1 protein manufactured in accordance with Example 5 was in each case incubated in a Preparation A with 5 mg of starch isolated from a *sex1-3* mutant of *Arabidopsis thaliana* in accordance with Example 1 b) and in a Preparation B with 5 mg of starch obtained by enzymatic phosphorylation in accordance with Example 1 c), in each case in 500 µl of phosphorylation buffer containing 0.05 mM radioactively (^{33}P) labeled, randomised ATP (in total 1,130,00 cpm, ca. 0.55 µCi) for 30 minutes at room temperature under agitation. A Preparation C which corresponded to the Preparation B but contained no OK1 protein but was otherwise treated in the same manner as Preparations A and B was used as control. For all the preparations (A, B, C) two tests were carried out independently of one another in each case.

15

Using a scintillation counter, the starches from Preparations A, B, and C were investigated for the presence of radioactively labeled phosphate (see Item 11 b), General Methods). The results are shown in Table 1 and in Fig. 3.

	Measured radioactivity [cpm]	
	Test 1	Test 2
Preparation A (non-phosphorylated starch + OK1)	42	47
Preparation B (phosphorylated starch + OK1)	7921	8226
Preparation C (phosphorylated starch without protein)	56	53

20 Table 1: Demonstration of starch-phosphorylating activity of the OK1 protein

From the results obtained, it can be seen that the OK1 protein does not transfer phosphate groups from ATP to starch when non-phosphorylated starch is provided

as a substrate, as the quota of phosphate groups transferred to non-phosphorylated starch by means of an OK1 protein, measured in cpm, does not exceed the quota of radioactively labeled phosphate groups in Preparation C (control). If, on the other hand, P-starch is provided as a substrate, the quota of radioactive phosphate groups, measured in cpm, which are transferred from ATP to P-starch, is significantly higher. From this, it can be seen that the OK1 protein requires P-starch as a substrate and that non-phosphorylated starch is not accepted as a substrate by the OK1 protein.

If the test described above is carried out with ATP specifically labeled in the gamma position with ^{33}P , then it is not possible to establish any incorporation of radioactively labeled phosphate in the starch. From this, it can be seen that the beta phosphate residue of ATP is transferred from an OK1 protein to starch. The results of such a test are shown in Fig. 6.

15 7. Demonstration of autophosphorylation

Autophosphorylation of the A.t.-OK1 protein was demonstrated by means of the methods described above (see Item 12, General Methods). Here, 50 µg of purified A.t.-OK1 protein were incubated with radioactively labeled, randomised ATP in 220 µl of phosphorylation buffer (see above, Item 12 d), General Methods) at room temperature for 60 minutes under agitation. Subsequently, 100 µl in each case was removed from the incubation preparations and transferred to four fresh reaction vessels. In reaction vessel 1, the reaction was stopped by the addition of 40 µl 0.11M EDTA. Reaction vessel 2 was incubated at 95°C for 5 minutes. HCl was added to reaction vessel 3 up to a final concentration of 0.5 M, and NaOH was added to reaction vessel 4 up to a final concentration of 0.5 M. Reaction vessels 3 and 4 were each incubated for 25 minutes at 30°C. Subsequently, 50 µl in each case was removed from reaction vessels 1, 2, 3 and 4, mixed with SDS test buffer and separated by means of SDS acrylamide gel electrophoresis (7.5% acrylamide gel). For this purpose, samples from the reaction vessels were applied to each of two identical acrylamide gels. One of the gels obtained on completion of electrophoresis

was subjected to autoradiography, while the second gel was stained with Coomassie Blue.

In the gel stained with Coomassie Blue (see Fig. 2A)), it can be clearly seen that
5 treatment with 0.5 M NaOH leads to degradation of the OK1 protein. The OK1 protein
must therefore be described as unstable towards NaOH. Incubations at 30°C, 95°C
and with 0.5 M HCl show that the OK1 protein is relatively stable under the stated
incubation conditions. This can be concluded from the fact that, under these
incubation conditions, in each case approximately the same amounts of OK1 protein
10 can be demonstrated in the gel concerned after staining with Coomassie Blue.

In the autoradiography (see Fig. 2B)), it can be seen by comparison with the
phosphorylated OK1 protein incubated at 30°C that an incubation of the
phosphorylated OK1 protein at 95°C leads to a significant reduction in the phosphate,
15 which has bound to the OK1 protein. The binding between the phosphate residue
and an amino acid of the OK1 protein must therefore be described as heat-unstable.
Furthermore, a slight reduction of the phosphate bound to the OK1 protein can also
be seen for the incubation with 0.5 M HCl and 0.5 M NaOH in comparison with
phosphorylated OK1 protein incubated at 30°C. If the fact is taken into account that
20 the quantity of OK1 protein in the autoradiography after treatment with 0.5 M NaOH
is significantly less than in the samples treated with heat and acid on account of the
instability of the OK1 protein towards NaOH, then it can be concluded that the
binding between the phosphate residue and an amino acid of the OK1 protein will be
relatively stable with respect to bases. As the sample treated with acid contains
25 approximately the same amounts of protein as the samples incubated at 30°C and at
95°C, and yet has a significantly lower signal in the autoradiography than the sample
treated at 30°C, it must be assumed that acid incubation conditions also split the
bond between a phosphate residue and an amino acid of the OK1 protein to a certain
extent. An instability of the binding between a phosphate residue and an amino acid
30 of the OK1 protein could therefore also be established in the tests carried out. At the

same time, the instability with respect to acids is significantly less labeled than the instability with respect to heat.

The binding between the amino acid histidine and phosphate are heat-unstable, acid-unstable but base-stable (Rosenberg, 1996, Protein Analysis and Purification, Birkhäuser, Boston, 242-244). The results described above are therefore an indication that a phosphohistidine is produced by the autophosphorylation of an OK1 protein.

10 If recombinantly expressed OK1 protein is incubated as described above with ATP specifically labeled with ^{33}P in the gamma position, no autophosphorylation can be established. Fig. 5 A) shows the amount of protein which can be detected in the respective reaction preparation by means of Western Blot analysis after the relevant incubation steps. Fig. 5 B) shows an autoradiography of protein from the individual
15 reaction preparations. It can be seen that, when ATP specifically labeled in the gamma position is used, no autophosphorylation of the OK1 protein can be demonstrated, whereas, when randomised ATP is used, autophosphorylation can be demonstrated. This means that when an OK1 protein is autophosphorylated, the phosphate residue of the beta position of the ATP is covalently bound to an amino
20 acid of the OK1 protein.

8. Demonstration of the C-atom positions, which are phosphorylated by an OK1 protein, of the glucose molecules of starch

a) Manufacture of phosphorylated starch

25 Phosphorylated starch was manufactured in accordance with Item 7, General Methods. To do this, 5 mg of non-phosphorylated starch, isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* was used in a Preparation A with 25 µg of purified A.t.-OK1 protein and, in a second Preparation B, 5 mg of *in vitro* phosphorylated starch originally isolated from leaves of a *sex1-3* mutant of
30 *Arabidopsis thaliana* was used with 5 µg of purified R1 protein. The reaction was

carried out in 500 μ l of phosphorylation buffer in each case, which, in each case contained ^{33}P labeled ATP (ca. 2.5×10^6 cpm), by incubating at room temperature for 1 hour under agitation. In addition, a control preparation was used, which contained 5 mg of starch isolated from leaves of a *sex1-3* mutant of *Arabidopsis thaliana* and the said phosphorylation buffer, but no protein. The control preparation was treated in exactly the same way as preparations A and B. The individual reactions were stopped by adding 125 μ l of 10% SDS in each case and washing was carried out once with 2% SDS, five times with 2 mM ATP and twice with H_2O , using 900 μ l in each case. Centrifugation was carried out after each washing step (2 minutes in an Eppendorf table centrifuge at 13,000 rpm in each case). The starch pellets obtained were resuspended in 1 ml H_2O in each case and 100 μ l of each preparation was mixed after the addition of 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN) and subsequently measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM).

The measurement gave the following results:

Control:	63 cpm/100 μ L	630 cpm/1000 μ l
Preparation A (OK1):	1351 cpm/100 μ l	13512 cpm/1000 μ l
Preparation B (R1):	3853 cpm/100 μ l	38526 cpm/1000 μ l

20

b) Total hydrolysis of the P-starch.

The suspensions of Preparations A, B and C obtained in accordance with Step a) were centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), the pellets obtained resuspended in 90 μ l 0.7 M HCl (Baker, for analysis) and subsequently incubated for 2 hours at 95°C. Preparations A, B and C were then centrifuged again (5 minutes in an Eppendorf table centrifuge at 13,000 rpm), and the supernatant transferred to a new reaction vessel. Sedimented residues of the preparations were resuspended in 100 ml H_2O in each case and after the addition of 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN) were measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN

30

COULTER™). Significant amounts of radioactivity could not be demonstrated in any of the residues, which means that all the hydrolysis products labeled with radioactive phosphate are located in the supernatant.

- 5 This was followed by neutralisation of the individual supernatants containing the hydrolysis products by the addition in each case of 30 µl 2 M NaOH (the amount of NaOH required for neutralisation was tested out in advance on blank samples). The neutralised hydrolysis products were placed on a 10 kDa Microcon filter, which had previously been rinsed twice with 200 µl H₂O in each case, and centrifuged for ca. 25
 10 minutes at 12,000 rpm in an Eppendorf table centrifuge. 10 µl was taken from the filtrate obtained (ca. 120 µl in each case) and, after the addition of 3 ml of scintillation cocktail (Ready-Safe™, BECKMANN), were measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM). The determination of the activity present in the individual preparations gave the following
 15 results:

Preparation A (OK1):	934 cpm/10 µl	11,208 cpm/120 µl	
	93 cpm/µl		
Preparation B (R1):	2518 cpm/10 µl	30,216 cpm/120 µl	252
			cpm/µl

20

c) Separation of the hydrolysis products

- The hydrolysis products obtained in accordance with Step b) were separated by means of HPAE using a Dionex system under the conditions stated above (see General Methods, Item 13 c)). The samples for separating the filtered supernatants
 25 of Preparations A and B obtained in accordance with Step b) were composed as follows:

Preparation A (OK1): 43 µl of the supernatant of Preparation A obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 32 µl H₂O, 2.5 µl 2.5 mM glucose-6-phosphate and 2.5 µl 5 mM glucose-3-phosphate (Σ Volume = 80 µl).

Preparation B (R1): 16 μ l of the supernatant of Preparation B obtained in accordance with Step b) (equivalent to ca. 4,000 cpm), 59 μ l H₂O, 2.5 μ l 2.5 mM glucose-6-phosphate and 2.5 μ l 5 mM glucose-3-phosphate (Σ Volume = 80 μ l).

- 5 In each case 60 μ l, containing ca. 3,000 cpm, of the corresponding samples was injected for separation using HPAE. The HPAE was carried out in accordance with the conditions specified under Point 23 c). After passing through the HPAE column, the elution buffer was collected in fractions, each of 1 ml. Collection of the fractions was begun 10 minutes after injecting the sample. Based on the signal received from
- 10 the PAD detector used, the elution of glucose-6-phosphate was assigned to fraction 15 and the elution of glucose-3-phosphate to fraction 17. In each case, 500 μ l of the individual fractions were mixed with 3 ml of scintillation cocktail (Ready SafeTM, BECKMANN) and subsequently measured using a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, BECKMANN COULTERTM). The following
- 15 measurements were obtained for the individual fractions:

Total cpm per fraction		
	Preparation A (OK1)	Preparation B (R1)
Fr 13	8.7	3.3
Fr 14	13.1	32.2
Fr 15 (G6P)	207.3	1952.8
Fr 16	3998	112.3
Fr 17 (G3P)	1749.2	801.6
Fr 18	196.7	17.3
Fr 19	6.7	18.9
Total	2581.5	2938.3
Deposit	3000.0	3000.0

Recovery	86.0%	97.9%
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Table 4: Measured amounts of radioactivity [cpm] in individual fractions of hydrolysis products obtained by hydrolysis of starch phosphorylated by means of an OK1 protein or R1 protein.

The results are also shown graphically in Fig. 5.

5

After phosphorylation of starch catalysed by R1 protein, ca. 66% of the radioactively labeled phosphate, referred to the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction which contained glucose-6-phosphate as standard, and ca. 27% with the fraction which
 10 contained glucose-3-phosphate as standard. After phosphorylation of starch catalysed by OK1 protein, ca. 67% of the radioactively labeled phosphate, referred to the total measured radioactive phosphate in the analysed fractions, eluted after hydrolysing the starch with the fraction which contained glucose-3-phosphate as standard, and ca. 8% with the fraction which contained glucose-6-phosphate as
 15 standard. From this, it can be concluded that glucose molecules of the starch of R1 proteins are preferably phosphorylated in the C-6 position, whereas from OK1 proteins glucose molecules of the starch are preferably phosphorylated in the C-3 position.

20 9. Identification of an OK1 protein in rice

Using the methods described under Items 1 to 13, General Methods, it was also possible to identify a protein from *Oryza sativa* (variety M202), which transfers a phosphate residue from ATP to P-starch. The protein was designated as O.s.-OK1. Non-phosphorylated starch is not used by the O.s.-OK1 protein as a substrate, i.e.
 25 the O.s.-OK1 protein also does need P-starch as a substrate. The nucleic acid sequence defining the identified O.s.-OK1 protein is shown under SEQ ID NO 3 and the amino acid sequence coding for the O.s.-OK1 protein is shown under SEQ ID NO. 4. The amino acid sequence coding for the O.s.-OK1 protein shown under SEQ ID NO 4 has an identity of 57% with the amino acid sequence coding for the A.t.-OK1

protein shown under SEQ ID NO 2. The nucleic acid sequence coding for the O.s.-OK1 protein shown under SEQ ID NO 3 has an identity of 61% with the nucleic acid sequence coding for the A.t.-OK1 protein shown under SEQ ID NO 1.

- 5 Manufacture of the plasmid pMI50 containing the nucleic acid sequence coding for an OK1 protein from *Oryza sativa*

The vector pMI50 contains a DNA fragment, which codes for the complete OK1 protein from rice of the variety M202.

The amplification of the DNA from rice was carried out in five sub-steps.

- 10 The part of the open reading frame from position -11 to position 288 of the sequence specified under SEQ ID NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-R9 (GGAACCGATAATGCCTACATGCTC) and Os_ok1-F6 (AAAACTCGAGGAGGATCAATGACGTCGCTGCGGCCCTC) as a primer on RNA of
- 15 immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML123.

- 20 The part of the open reading frame from position 250 to position 949 of the sequence specified under SEQ ID NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F4 (CCAGGTAAAGTTTGGTGAGCA) and Os_ok1-R6 (CAAAGCACGATATCTGACCTGT) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained
- 25 was designated as pML120.

- The part of the open reading frame from position 839 to position 1761 of the sequence specified under SEQ ID NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F7
- 30 (TTGTTTCGCGGATATTGTCAGA) and Os_ok1-R7 (GACAAGGGCATCAAGAGTAGTATC)

as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML121.

- 5 The part of the open reading frame from position 1571 to position 3241 of the sequence specified under SEQ DIE NO 3 was amplified using reverse transcriptase and polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F8 (ATGATGCGCCTGATAATGCT) and Os_ok1-R4 (GGCAAACAGTATGAAGCACGA) as a primer on RNA of immature rice seeds. The amplified DNA fragment was cloned into
10 the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML119.

- The part of the open reading frame from position 2777 to position 3621 was amplified using polymerase chain reaction using the synthetic oligonucleotides Os_ok1-F3
15 (CATTGGATCAATGGAGGATG) and Os_ok1-R2 (CTATGGCTGTGGCCTGCTTTGCA) as a primer on genomic DNA of rice. The amplified DNA fragment was cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pML122.

- 20 The cloning together of the sub-parts of the open reading frame of OK1 was carried out as follows.

A 700 base pair long *Apal* fragment of pML120, containing part of the open reading frame of OK1, was cloned in the *Apal* site of pML121. The plasmid obtained was designated as pML147.

25

- A 960 base pair long fragment containing the regions of the vectors from pML120 and pML123 coding for OK1 was amplified by means of polymerase chain reaction. In doing so, the primers Os_ok1-F4 (see above) and Os_ok1-R9 (see above), each in a concentration of 50 nm, and the primers Os_ok1-F6 and Os_ok1-R6, each in a
30 concentration of 500 nm, were used. The amplified DNA fragment was cloned into

the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as pMI44.

5 An 845 base pair long fragment of pML122 was reamplified for introducing a *Xho*I site after the stop codon with the primers Os_Ork1-F3 (see above) and Os_ork1-R2Xho (AAAACTCGAGCTATGGCTGTGGCCTGCTTTGCA) and cloned into the vector pCR2.1 (Invitrogen catalogue number K2020-20). The plasmid obtained was designated as t pMI45.

10 A 1671 base pair long fragment containing part of the open reading frame of OK1 was obtained from pML119 by digesting with the restriction enzymes *Spe*I and *Pst*I. The fragment was cloned into pBluescript II SK+ (Genbank Acc.: X52328). The plasmid obtained was designated as pMI46.

15 A 1706 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Spe*I and *Xho*I from pMI46 and cloned into the vector pMI45, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI47.

20 A 146 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Afl*II/*Not*I from pMI43 and cloned into the vector pMI44, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI49.

25 A 1657 base pair long fragment containing part of the open reading frame of OK1 was excised with the restriction enzymes *Not*I and *Nar*I from the vector pMI49 and cloned into the vector pMI47, which had been excised with the same restriction enzymes. The plasmid obtained was designated as pMI50 and contains the whole coding region of the OK1 protein identified in rice.

10. Identification of further OK1 proteins from various plant species

Using the methods described under Items 1 to 13, General Methods, proteins which transfer a phosphate residue from ATP to P-starch were also identified in barley
5 (*Hordeum vulgare*), potato (*Solanum tuberosum*), wheat (*Triticum aestivum*) and millet (*Sorghum bicolor*). Non-phosphorylated starch is not used as substrate by these proteins, i.e., these proteins require P-starch as substrate.

The proteins were isolated using the method described under Item 14, General
10 Methods, digested with trypsin, dissolved out of the gel and sequenced using Q-TOF-MS-MS. Using the peptide sequences obtained, it was possible to determine EST nucleic acid sequences which code for the relevant OK1 proteins from barley, potato, wheat or millet by means of database comparisons (blast searches).

15 The nucleic acid sequence shown in SEQ ID NO 9 codes for a part of an OK1 protein from barley and was traced under "Accession" No.: TC117610 in the TIGR (<http://tigrblast.tigr.org/tgi/>) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from barley using Q-TOF-MS-MS and were used to identify the EST nucleic
20 acid sequence shown under SEQ ID NO 9, are specified in SEQ ID NO 6, SEQ ID NO 7 and SEQ ID NO 8. The amino acid sequence shown in SEQ ID NO 10 codes for a part of an OK1 protein from barley and can be derived from the nucleic acid sequence shown in SEQ ID NO 10.

25 The nucleic acid sequence shown in SEQ ID NO 15 codes for a part of an OK1 protein from potato and was found under "Accession" No.: BFO54632 in the TIGR (<http://tigrblast.tigr.org/tgi/>) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from potato using Q-TOF-MS-MS and were used to identify the EST nucleic
30 acid sequence shown under SEQ ID NO 15, are specified in SEQ ID NO 11, SEQ ID

NO 12, SEQ ID NO 13 and SEQ ID NO 14. The amino acid sequence shown in SEQ ID NO 16 codes for a part of an OK1 protein from potato and can be derived from the nucleic acid sequence shown in SEQ ID NO 15.

- 5 The nucleic acid sequence shown in SEQ ID NO 21 codes for a part of an OK1 protein from millet and was found under "Accession" No.: TC77219 in the TIGR (<http://tigrblast.tigr.org/tgi/>) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from millet using Q-TOF-MS-MS and were used to identify the EST nucleic acid sequence shown under SEQ ID NO 21, are specified in SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19 and SEQ ID NO 20. The amino acid sequence shown in SEQ ID NO 22 codes for a part of an OK1 protein from millet and can be derived from the nucleic acid sequence shown in SEQ ID NO 21.
- 10
- 15 The nucleic acid sequence shown in SEQ ID NO 25 codes for a part of an OK1 protein from wheat and was found under "Accession" No.: CA74319 in the TIGR (<http://tigrblast.tigr.org/tgi/>) database by means of a database comparison (blast search). Those peptides which were obtained by sequencing the OK1 protein isolated from wheat using Q-TOF-MS-MS and were used to identify the EST nucleic acid sequence shown under SEQ ID NO 25, are specified in SEQ ID NO 23 and SEQ ID NO 24. The amino acid sequence shown in SEQ ID NO 26 codes for a part of an OK1 protein from wheat and can be derived from the nucleic acid sequence shown in SEQ ID NO 25.
- 20

The following settings were selected to carry out the database comparisons:

- 25 Program: tblastn
- Matrix: blosum62
- Expect: 100
- Echofilter: disabled
- Descriptions: 20

All other settings read "default".

11. Manufacture of an antibody, which specifically recognises an OK1 protein

As an antigen, ca. 100 µg of purified A.t.-OK1 protein was separated by means of
 5 SDS gel electrophoresis, the protein bands containing the A.t.-OK1 protein excised
 and sent to the company EUROGENTEC S.A. (Belgium), which carried out the
 manufacture of the antibody under contract. First, the preimmune sera of rabbits
 were investigated to see whether they would already recognise a protein from an A. t.
 total extract before immunisation with recombinant OK1. The preimmune sera of two
 10 rabbits recognised no proteins in the range 100-150 kDa and were thus chosen for
 immunisation. Four injections of 100 µg of protein (day 0, 14, 28, 56) were given to
 each rabbit. Four blood samples were taken from each rabbit: (day 38, day 66, day
 87 and the final bleeding). Serum obtained after the first bleeding, already showed a
 specific reaction with OK1 antigen in Western blot. However, in all further tests, the
 15 final bleeding of a rabbit was used

12. Manufacture of transgenic rice plants which have an elevated or a reduced activity of an OK1 protein

a) Manufacture of the plasmid pGlo-A.t.-OK1

The plasmid pIR94 was obtained by amplifying the promoter of the globulin gene
 20 from rice by means of a polymerase chain reaction (30 x 20 sec 94 °C, 20 sec 62 °C,
 1 min 68 °C, 4 mM Mg2SO4) with the primers glb1-F2
 (AAAACAATTGGCGCCTGGAGGGAGGAGA) and glb1-R1
 (AAAACAATTGATGATCAATCAGACAATCACTAGAA) on the genomic DNA of rice
 of the variety M202 with High Fidelity Taq Polymerase (Invitrogen, catalogue number
 25 11304-011) and cloned into pCR2.1 (Invitrogen catalogue number K2020-20).

The plasmid pIR115 was obtained by cloning a synthetic piece of DNA consisting of
 the two oligonucleotides X1
 (TGCAGGCTGCAGAGCTCCTAGGCTCGAGTTAACACTAGTAAGCTTAATTAAGAT
 ATCATTTAC) and X2

(AATTGTAAATGATATCTTAATTAAGCTTACTAGTGTTAACTCGAGCCTAGGAGCTCTGCAGCCTGCA) into the vector pGSV71 excised with *SdaI* and *MunI*.

The plasmid pIR115 obtained was excised with *SdaI*, the protruding 3'-ends smoothed with T4 DNA polymerase and a 197-base-pair *HindIII* / *SphI* fragment from pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230), smoothed by
5 means of T4 DNA polymerase and containing the termination signal of the octopine synthase gene from *Agrobacterium tumefaciens*, was inserted. The plasmid obtained was designated as pIR96.

10 The plasmid pIR103 was obtained by cloning a 986-base-pair long DNA fragment from pIR94, containing the promoter of the globulin gene from rice, into the plasmid pIR96.

pGSV71 is a derivative of the plasmid pGSV7, which is derived from the intermediary
15 vector pGSV1. pGSV1 is a derivative of pGSC1700 whose construction has been described by Cornelissen and Vanderwiele (Nucleic Acid Research 17, (1989), 19-25). pGSV1 was obtained from pGSC1700 by deletion of the carbenicillin resistance gene, as well as deletion of the T-DNA sequences of the TL-DNA region of the plasmid pTiB6S3.

20 pGSV7 contains the replication origin of the plasmid pBR322 (Bolivar et al., Gene 2, (1977), 95-113) as well as the replication origin of the *Pseudomonas* plasmid pVS1 (Itoh et al., Plasmid 11, (1984), 206). pGSV7 also contains the selectable marker gene *aadA*, from the transposon Tn1331 from *Klebsiella pneumoniae*, which imparts resistance to the antibiotics spectinomycin and streptomycin (Tolmasky, Plasmid 24
25 (3), (1990), 218-226; Tolmasky and Crosa, Plasmid 29(1), (1993), 31-40)

The plasmid pGSV71 was obtained by cloning a chimeric *bar* gene between the border regions of pGSV7. The chimeric *bar* gene contains the promoter sequence of the cauliflower mosaic virus for initiation of the transcription (Odell et al., Nature 313,
30 (1985), 180), the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al.,

Embo J. 6, (1987), 2519-2523) and the 3'-untranslated region of the nopaline synthase gene of the T-DNA of pTIT37 for termination of the transcription and polyadenylation. The *bar* gene provides tolerance against the herbicide glufosinate ammonium.

5

A DNA fragment which contains the complete open reading frame of the OK1 protein from *Arabidopsis* was excised from the vector A.t.-ok1-pGEM-T and cloned into the vector pIR103. For this purpose the plasmid A.t.-OK1-pGEM-T was excised with the restriction enzyme *Bsp120I*, the ends smoothed with T4-DNA polymerase and subsequently excised with *SaII*. The DNA fragment coding for the OK1 protein from *Arabidopsis thaliana* was cloned into the vector pIR103 excised with *Ecl136II* and *XhoI*. The plasmid obtained was designated as pGlo-A.t.-OK1.

b) Manufacture of a construct for inhibiting the OK1 protein in rice by means of RNAi technology

The plasmid pML125, which was used for the transformation of rice plants, was obtained by specific recombination of the plasmids pML124 and pIR115 using the Gateway™ cloning system (Invitrogen).

pML124 was obtained by cloning a 359 base pair long DNA fragment of pML119 (see above, Example 9), containing part of the open reading frame which codes for the OK1 protein from rice, into the vector pENTR-1A (Invitrogen, product number 11813-011) excised with *EcoRI*.

The plasmid pIR87 was obtained by amplifying the intron 1 of the gene coding for alcohol hydrogenase from maize with the primers Adh(i)-1 (TTTCTCGAGGTCCGCCTTGTTCTCCT) and Adh(i)-2 (TTTCTCGAGCTGCACGGGTCCAGGA) on the genomic DNA of maize. The product of the polymerase chain reaction (30 x 30 sec 94 °C, 30 sec 59 °C, 1 min 72 °C, 2.5 mM MgCl₂) was digested with the restriction enzyme *XhoI* and cloned into the vector

pBluescript II SK+ (Genbank Acc.: X52328), which had been excised with the same enzyme.

5 A 986 base pair long DNA fragment from pIR94, containing the promoter of the globulin gene from rice, was cloned into the vector pIR96. The plasmid obtained was designated as pIR103.

The plasmid pIR107 was obtained by cloning the "RfA cassette" (see above) into the plasmid pIR103 excised with the restriction enzyme *EcoRV*.

10

A 540 base pair long fragment containing the intron 1 of the gene coding for alcohol dehydrogenase from maize was excised from the plasmid pIR87 with the restriction enzyme *XhoI* and cloned into the plasmid pIR107 likewise excised with *XhoI*. The plasmid obtained was designated as pIR114. The plasmid pIR115 was obtained by
15 cloning the "RfA cassette" (see above) into the plasmid pIR114 excised with *Ecl136II*.

c) Transformation of rice plants

Rice plants (variety M202) were transformed using *Agrobacterium* (containing either the plasmid pGlo-A.t.-OK1 or the plasmid pML125) using the method described in
20 Hiei et al. (1994, Plant Journal 6(2), 271-282).

d) Analysis of the transgenic rice plants which expressed the A.t.-OK1 protein and the starch synthesised by these plants

Plants transformed with the plasmid pGlo-A.t.-OK1 which exhibited an expression of
25 the heterologous A.t.-OK1 protein were identified by means of a Northern Blot analysis.

Plants which exhibited a detectable quantity of mRNA coding for A.t.-OK1 protein were cultivated in the greenhouse. Grains of these plants were harvested. Starch from these grains showed an elevated content of phosphate covalently bound to the starch concerned.

5

e) Analysis of the transgenic rice plants in which the expression of the endogenous OK1 protein was repressed by means of RNAi technology and the starch synthesised from these plants

Rice plants which were transformed with the plasmid pML125 and exhibited a reduced expression of the endogenous mRNA coding for the OK1 protein were identified by means of Northern Blot analysis.

13. Manufacture of transgenic potato plants which have an elevated or a reduced activity of an OK1 protein

15 a) Manufacture of the plasmid pBinB33-Hyg

Starting from the plasmid pBinB33, the *EcoRI-HindIII* fragment including the B33 promoter, a part of the polylinker, and the *ocs* terminator were excised and ligated into the correspondingly excised vector pBIB-Hyg (Becker, 1990, Nucl. Acids Res. 18, 203). Acids Res. 18, 203).

20

The plasmid pBinB33 was obtained by ligating the promoter of the patatin gene B33 from *Solanum tuberosum* (Rocha-Sosa et al., 1989) as a *DraI* fragment (nucleotide – 1512 - +14) into the vector pUC19 excised with *SstI*, the ends of which had been smoothed using the T4 DNA polymerase. This resulted in the plasmid pUC19-B33.

25 The B33 promoter was excised from this plasmid with *EcoRI* and *SmaI* and ligated into the correspondingly excised vector pBinAR (Höfgen and Willmitzer, 1990, Plant Science 66, 221-230). This resulted in the plant expression vector pBinB33.

b) Manufacture of the vector A.t.-OK1-pBinB33-Hyg

The coding sequence of the A.t.-OK1 protein was excised with the restriction endonucleases *Bsp120I* and *Sall* from the plasmid OK1-pGEM and ligated into the vector pBinB33-Hyg excised with *SmaI* and *Sall*. The plasmid obtained was designated as A.t.-OK1-pBinB33-Hyg.

5

c) Transformation of potato plants

Agrobacterium tumefaciens (strain GV2260) was transformed with the plasmid A.t.-OK1-pBinB33-Hyg. Potato plants of the Désirée variety were then transformed using agrobacteria containing the plasmid A.t.-OK1-pBinB33-Hyg using the method described in Rocha-Sosa et al. (EMBO J. 8, (1989), 23-29) and plants regenerated. The plants obtained from this transformation event were designated 385JH.

10

d) Analysis of the transgenic potato plants and the starch synthesised by these

Plants which exhibited an elevated activity of the heterologously expressed A.t.-OK1 protein and also plants in which the activity of the endogenous OK1 protein was reduced by a co-suppression effect were identified by means of a Western Blot analysis. The Western Blot analysis was carried out using the antibody described under Example 11

20

Fig. 7 exemplary shows the detection of the A.t.-OK1 Protein in single plants from the transformation event 385JH by means of Western Blot analysis. For induction of the B33 Promotor in leaf tissue single lines of the the transformation event 385JH were cultivated on solidified Musharige Skoog medium containing 100 mM sucrose in tissue culture for two days. After harvest protein extracts were produced from leaf tissue of these plants according to the method described under General Methods, Item 1a).. After separation of the proteins by means of denaturing polyacrylamide gel electrophoreses 40 µg protein extract of each line was analysed by means of Western Blot analysis using the antibody described under Examples, Item 10. As

25

control samples, protein extracts from *Arabidopsis* plants and from potato wildtype plants (cv Désirée) were also analysed.

Plants which exhibited an elevated quantity of A.t.-OK1 protein compared to the
5 corresponding wild type plants were cultivated in the greenhouse. Starch which was isolated from tubers of these plants showed an elevated content of phosphate covalently bound to the starch compared to the starch isolated from non-transformed wild type plants.

10 **14. Analysis of *Arabidopsis thaliana* plants which exhibit a reduced activity of a protein according to the invention**

T-DNA insertion mutants of *Arabidopsis thaliana* (available from the Salk Institute Genomic Analysis Laboratory, 10010 N. Torrey Pines Road, La Jolla, CA 92037, <http://signal.salk.edu/> under ACC. No.: Salk_110814, Alias N610814), which were
15 homozygotic with respect to insertion in the OK1 gene, were grown under the following conditions:

Light phase: 16 hours, 20°C

Dark phase: 8 hours, 16°C

Shortly before the flowers developed, the plants were cultivated in a light phase of 12
20 hours at 20°C and a dark phase of 12 hours at 17°C.

Plants of the mutant line obtained (Salk_110814) were cultivated from 3 different seeds of the original seed material (Salk_110814-1, Salk_110814-2, Salk_110814-3) for analysis.

25

At the end of the dark phase, 10 leaves were removed in each case from 6 wild type plants (Ökotyp Columbia) and decolourised in 70% ethanol at 50°C. Furthermore, 6 leaves were removed in each case from respectively 4 different plants of the mutant

lines Salk_110814-1, Salk_110814-2 or Salk_110814-3 which were in each case homozygotic with respect to T-DNA insertion in an OK1 gene, and these were decolourised in 70% ethanol at 50°C. The leaves were then incubated for 10 minutes in Lugol's solution before excess Lugol's solution was rinsed off the leaves with tap
5 water. All leaves from wild type plants showed no staining with Lugol's solution. On the other hand, all leaves of the mutant lines Salk_110814-1, Salk_110814-2 or Salk_110814-3 showed a dark brown or black colouration (see Fig. 7). The mutant lines therefore showed a starch excess phenotype compared to the wild type plants. During cultivation no differences relating to the growth could be established between
10 the mutant lines and the wild type plants.

Genetically modified *Arabidopsis thaliana* plants which were transformed with an RNAi construct containing "inverted repeats" of the coding region of an OK1 gene under control of the 35S promoter, were analysed with the aid of Western blot analysis using the antibody described in Example 10. Several independent lines
15 which exhibited a reduced quantity of OK1 protein compared to wild type plants were identified. These lines were cultivated under the culture conditions specified above. In each case, 5 leaves of the individual lines were removed at the end of the dark phase (12 hours at 17°C), decolourised in ethanol and stained with Lugol's solution. All the plants showed a starch excess phenotype compared to corresponding wild
20 type plants. During cultivation no differences relating to growth could be established between the genetically modified plants and the wild type plants. The plants genetically modified by means of RNAi technology thus showed the same properties as the mutant lines Salk_110814-1, Salk_110814-2 or Salk_110814-3.

25 In each case four *Arabidopsis thaliana* plants of the lines A.t.-alpha-OK1-1, A.t.-alpha-OK1-2, A.t.-alpha-OK1-3, A.t.-alpha-OK1-4, A.t.-alpha-OK1-5, resulting from independent transformation events, in which the quantity of OK1 protein is reduced by means of RNAi technology, were investigated for their starch content at different times. The reduction in the quantity of OK1 protein in the respective lines was
30 demonstrated by means of Western blot analysis (see Fig. 8). The leaf starch content of the individual lines was determined using the starch kits from Boehringer Mannheim (Product No.: 0207748). For this purpose, in each case all the leaves of

four plants of the individual lines were harvested and the leaves were homogenised using mortars. 40 mg to 60 mg of the homogenised leaf material was washed twice with 80% ethanol in each case and the supernatant was discarded. The remaining material, which is not soluble in ethanol, was freeze-dried after being washed once in
 5 1 ml of water, then dissolved in 0.5 ml of 0.2M KOH at 95°C for 1 h and the solution obtained was adjusted to pH 7 using 88 µL of 1M acetic acid. 25 µl of the respective solution obtained was mixed with 50 µl of amyloglucosidase solution (Starch-Kit from Boehringer Mannheim, Product No.: 0207748), to which 1 unit of alpha-amylase (from *Bacillus amyloliquefaciens*, Boehringer, Prod-No. 161764) had been added and
 10 was incubated for 1 h at 55 °C. 20 µl of the solution treated with amyloglucosidase and alpha-amylase was then used to determine the glucose using an enzymatic coupled photometric test (see product information sheet for the determination of native starch from Boehringer Ingelheim, Product No.: 0207748) At the same time as the transgenic lines, the starch content was also determined in leaves of
 15 *Arabidopsis thaliana* wild type plants (Ecotype Columbia). The wild type plants and the transgenic plants were cultivated under the same conditions: 12 hours light phase followed by 12 hours dark phase.

Leaves of the respective transgenic plant lines and wild type plants were harvested in
 20 each case ca. 4.5 weeks after seed germination after the end of the dark phase, after the end of a light phase and after the end of a second dark phase which directly followed the light phase. For each transgenic plant line, two independent extracts were produced in each case, from which two measurements of the starch content were made in each case. For wild type plants four extracts were produced in each
 25 case from which two measurements of the starch content were made in each case. The determination of the leaf starch contents yielded the following results:

Starch content		
Line	(mg/g FW)	Standard deviation*

		Starch content	
		(mg/g FW)	Standard deviation*
End			
dark phase 1			
	A.t.-alpha-OK1-1	4.09	0.55
	A.t.-alpha-OK1-2	4.93	0.94
	A.t.-alpha-OK1-3	5.59	0.52
	A.t.-alpha-OK1-4	6.36	0.87
	A.t.-alpha-OK1-5	1.49	0.99
	Wild type	0.78	0.14
End			
light phase			
	A.t.-alpha-OK1-1	9.30	0.96
	A.t.-alpha-OK1-2	9.86	1.45
	A.t.-alpha-OK1-3	11.68	1.60
	A.t.-alpha-OK1-4	9.53	1.25
	A.t.-alpha-OK1-5	6.61	0.71
	Wild type	5.61	0.72
End			
dark phase 2			
	A.t.-alpha-OK1-1	3.92	0.83
	A.t.-alpha-OK1-2	4.35	1.07
	A.t.-alpha-OK1-3	6.00	0.63

Line	Starch content	
	(mg/g FW)	Standard deviation*
A.t.-alpha-OK1-4	5.34	1.35
A.t.-alpha-OK1-5	1.46	0.56
Wild type	0.62	0.18

Table 4: Quantity of leaf starch in *Arabidopsis thaliana* plants in which the quantity of OK1 protein is reduced using RNAi technology.

* Standard deviation using the general formula: $\text{root} [(n\sum x^2 - (\sum x)^2) / n(n-1)]$

5

15. Analysis of starch isolated from plants which exhibit a reduced activity of an OK1 protein

Starch was isolated from leaves of the plants described in Example 14 and hydrolysed using the method described under General Methods, Item 13 and then separated by means of HPAE analysis. The areas of the separated signals obtained by means of HPAE analysis for C-3 phosphate and C-6 phosphate were calculated (Software: Chromelion 6.20 from Dionex, USA) and the values obtained were given as the ratio to one another. The ratio of C-6 phosphate to C-3 phosphate in wild type plants was 2.1. In the plants described in Example 14 in which the activity of the OK1 protein was reduced by means of RNAi technology, on the other hand, the average ratio of C-6 phosphate to C-3 phosphate determined by analysing the starch isolated from the lines A.t.-alpha-OK1-1, A.t.-alpha-OK1-2, A.t.-alpha-OK1-3, A.t.-alpha-OK1-4 and A.t.-alpha-OK1-5 was 2.5. The analysis of starch from the line A.t.-alpha-OK1-5 yielded the lowest ratio of C-6 phosphate to C-3 phosphate (ratio of 2.2), starch from the line A.t.-alpha-OK1-1 yielded the highest ratio (ratio of 2.7).

Starch isolated from leaves of the mutants described in Example 13 which exhibit a reduced activity of an OK1 protein showed an increase in the ratio of C-6 phosphate to C-3 phosphate in the starch concerned.

5

10

Patent claims

1. A method for identifying a protein which has an elevated binding activity towards phosphorylated alpha-1,4-glucans, compared to non-phosphorylated alpha-1,4 glucans, wherein
 - a) protein extracts in preparations separate from one another are incubated with
 - i phosphorylated alpha-1,4-glucans and
 - ii non-phosphorylated alpha-1,4-glucans,
 - b) proteins specifically bound to the
 - i phosphorylated alpha-1,4-glucans from step a) i and
 - ii proteins specifically bound to the non-phosphorylated alpha-1,4-glucans from step a) iiare dissolved in preparations separate from one another and
 - c) proteins are identified which exhibit an elevated binding activity towards phosphorylated alpha-1,4-glucans used in step b) i, compared to non-phosphorylated alpha-1,4-glucans used in step b) ii.
2. A method for identifying a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity and requires phosphorylated alpha-1,4-glucans as substrate, wherein
 - a) protein extracts are incubated with phosphorylated alpha-1,4-glucans,
 - b) proteins specifically bound to the phosphorylated alpha-1,4-glucans from step a) are dissolved,
 - c) proteins obtained according to step b) are respectively incubated with
 - i ATP and phosphorylated alpha-1,4-glucans and
 - ii ATP and non-phosphorylated alpha-1,4-glucansin preparations separated from one another,
 - d) the respective alpha-1,4-glucan obtained after incubation in step c) i or step c) ii is examined for introduction of further phosphate groups and
 - e) proteins are identified which in the incubation preparation according to c) i have introduced significant quantities of phosphate groups into alpha-1,4-

glucans and in the incubation preparation according to c) ii have introduced no significant quantities of phosphate groups into alpha-1,4-glucans.

3. The method according to claim 2, wherein the protein with alpha-1,4-glucan phosphorylating enzymatic activity uses phosphorylated starch as substrate.
4. The method according to claim 3, wherein the protein with alpha-1,4-glucan phosphorylating enzymatic activity originates from a plant.
5. A protein obtainable by a method according to one of claims 1 to 4.
6. A method for identifying a nucleic acid molecule coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity, wherein
 - a) a protein is identified by a method according one of claims 1 to 4,
 - b) amino acid sequences coding for the protein identified according to step a) are determined and
 - c) nucleic acid molecules are identified using the amino acids determined according to step b).
7. The method according to claim 6, wherein nucleic acid oligonucleotides based on the amino acid sequence determined according to step b) are manufactured to identify said nucleic acid molecule according to step c).
8. The method for identifying a nucleic acid molecule coding for a protein which exhibits alpha-1,4-glucan phosphorylating enzymatic activity, wherein
 - a) a protein is identified by a method according one of claims 1 to 4,
 - b) antibodies which react specifically with the protein identified according to step a) are produced and
 - c) nucleic acid molecules are identified using the antibodies produced according to step b).
9. A nucleic acid molecule obtainable by a method according to one of claims 6, 7 or 8.
10. A genetically modified plant cell, characterised in that it exhibits an elevated enzymatic activity of a protein according to claim 5 or a protein which can be

obtained by a method according to one of claims 1 to 4 compared to corresponding wild type plant cells which have not been genetically modified.

11. The genetically modified plant cell according to claim 10 which is a maize, rice, wheat, rye, oats, barley, cassava, potato, sweet potato, sago, mung bean, banana, pea, Arabidopsis, curcuma or sorghum plant.
12. A genetically modified plant characterised in that the genetic modification consists in the introduction of at least one foreign nucleic acid molecule according to claim 9 or which can be obtained by a method according to one of claims 6, 7 or 8 into the genome of the plant.
13. The genetically modified plant cell according to claim 12, which synthesises a modified starch compared to starch from corresponding wild type plant cells.
14. The genetically modified plant cell according to claim 13, which synthesises a modified starch which has an elevated content of starch phosphate and/or a modified phosphate distribution compared to starch from corresponding wild type plants.
15. The plant cell according to claim 14, wherein the modified starch is characterised in that it exhibits an elevated content of phosphate covalently bound to the starch in the C-3 position of the glucose molecule compared to starch from corresponding wild type plant cells.
16. A plant containing genetically modified plant cells according to one of claims 10 to 15.
17. The plant according to claim 16, which is a maize, rice, wheat, rye, oat, barley, cassava, potato, sago, mung bean, pea or sorghum plant.
18. The plant according to claim 17, which is a maize or wheat plant.

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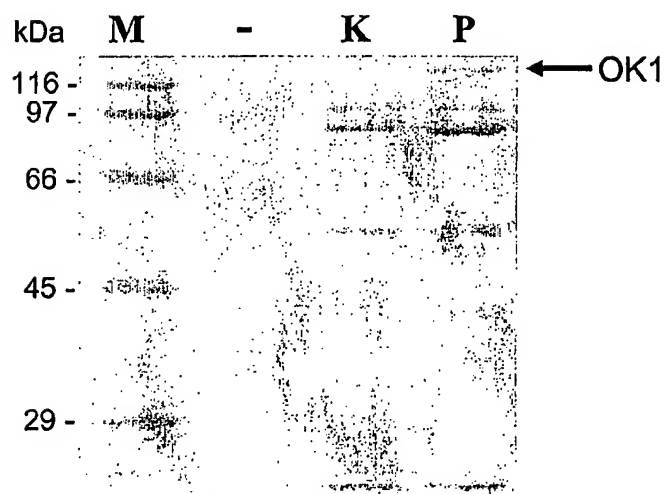


Fig. 1

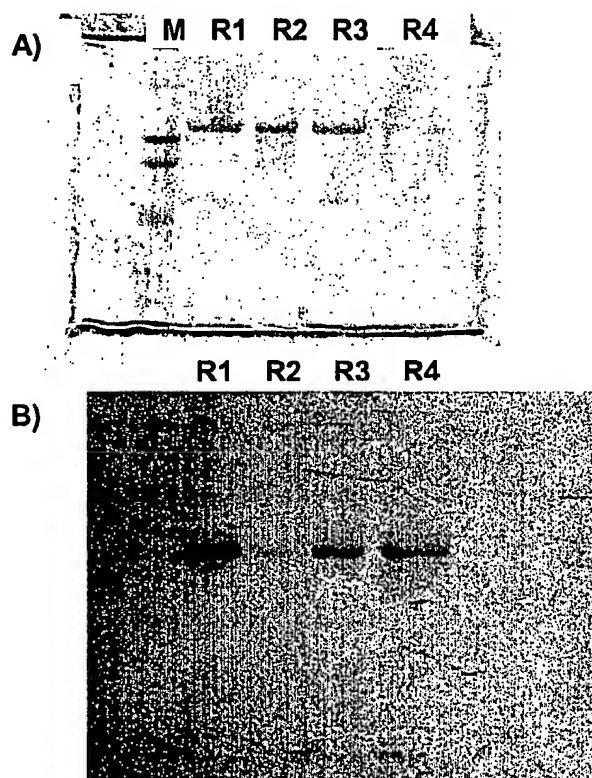


Fig. 2

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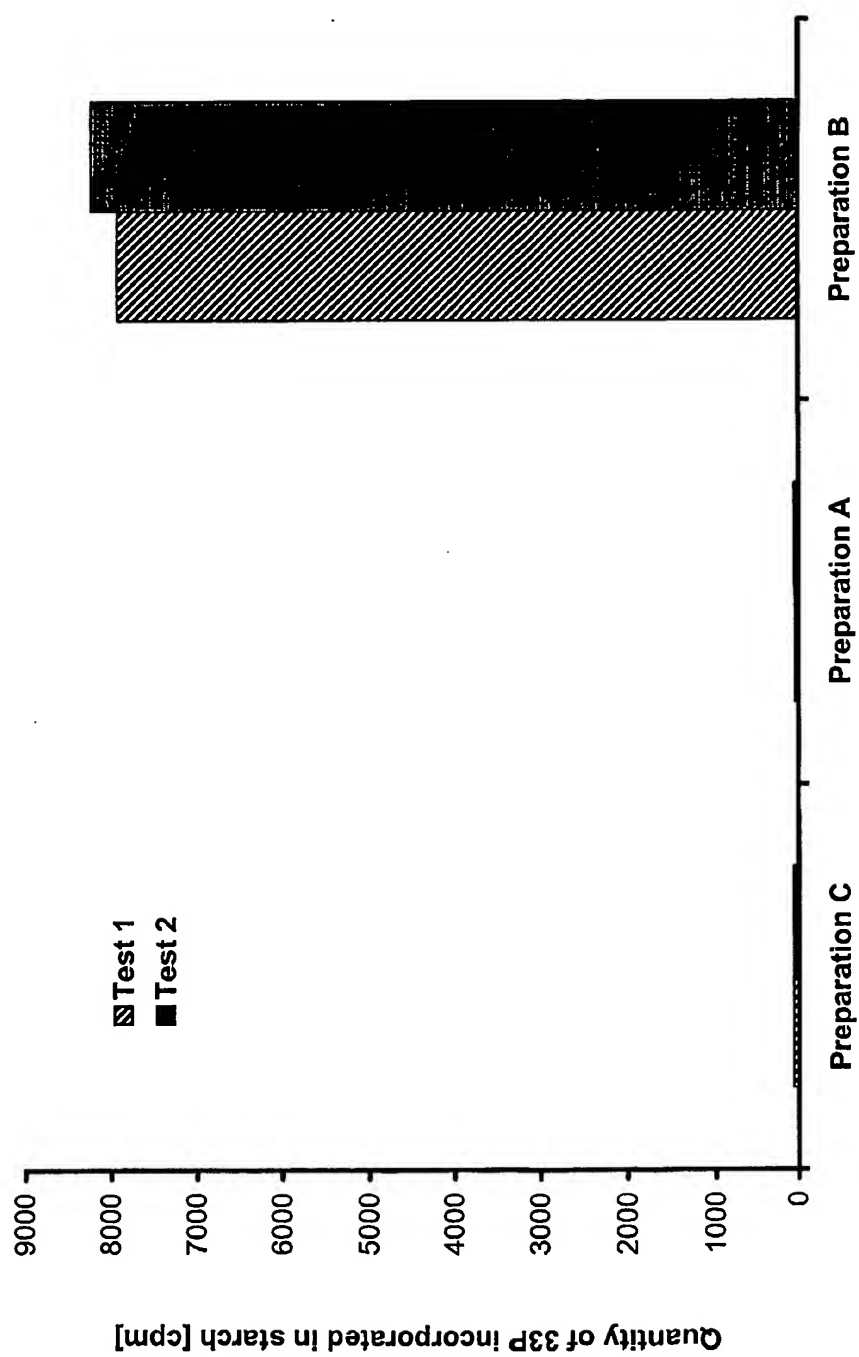


Fig.: 3

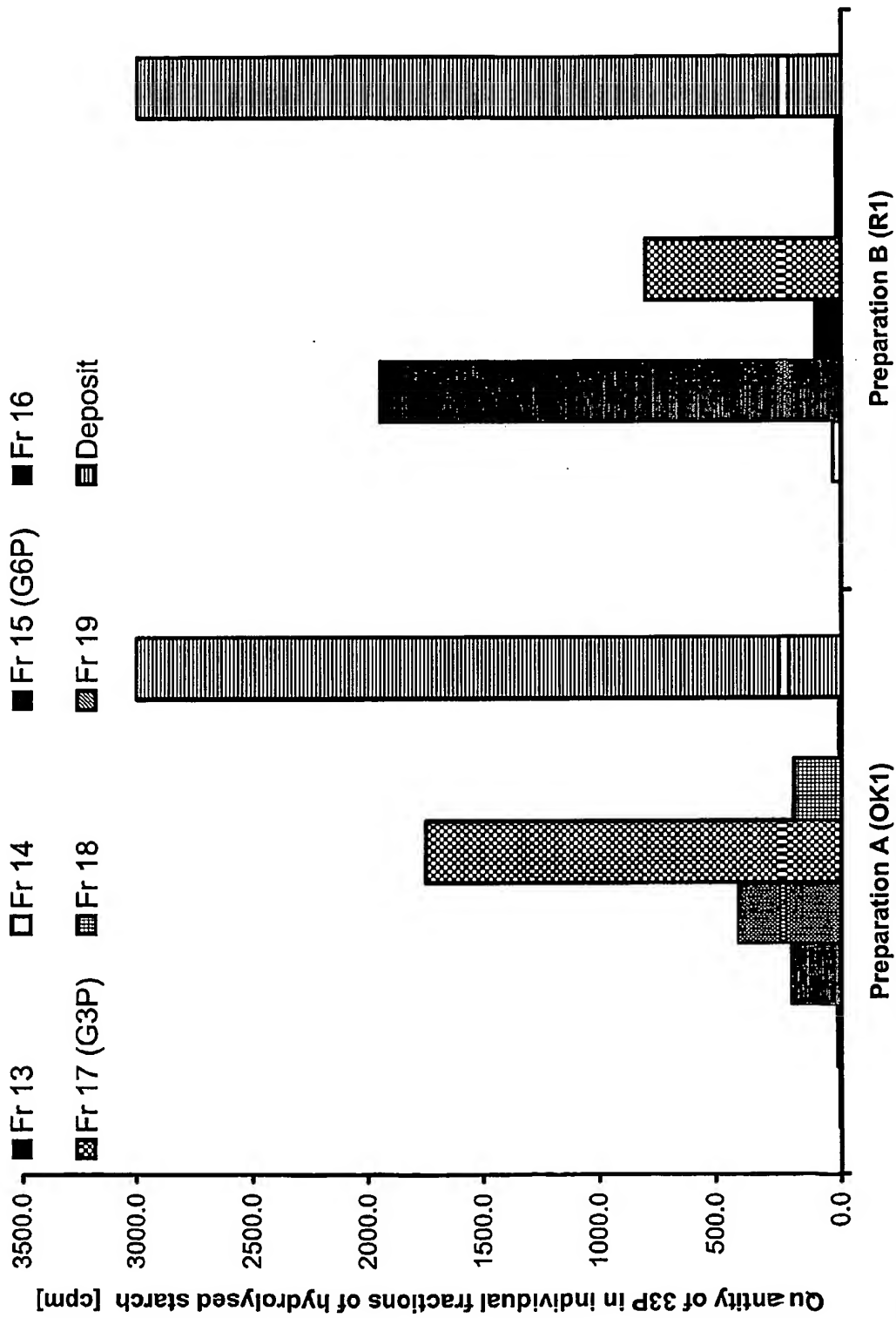


Fig. 4

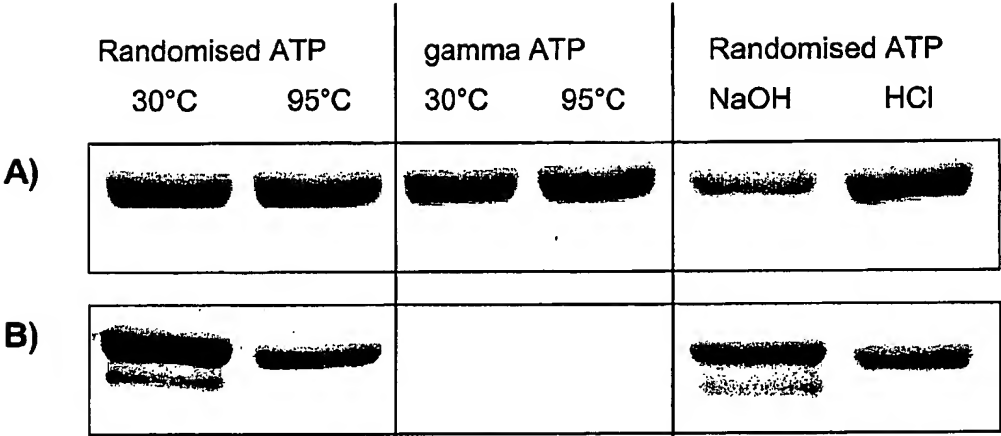


Fig. 5

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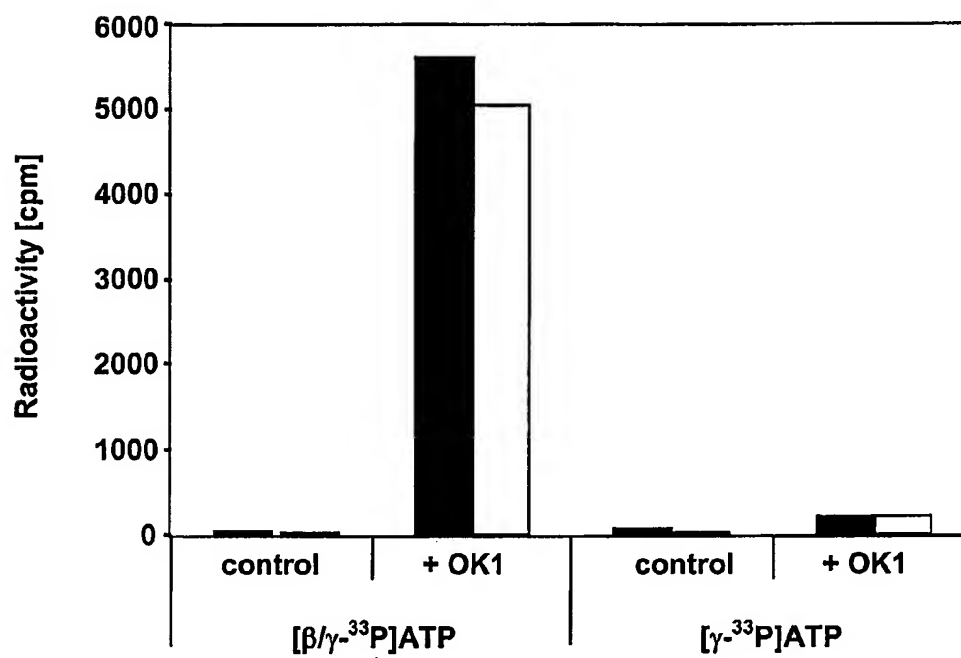


Fig. 6

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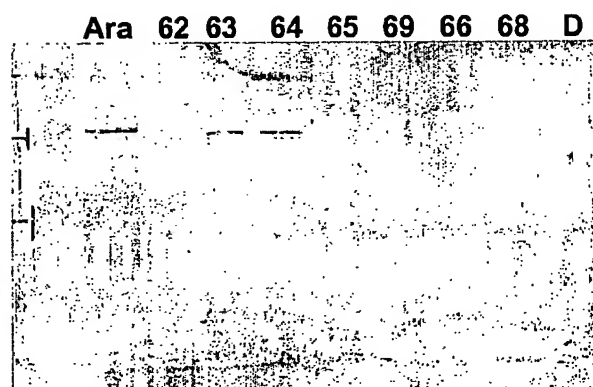
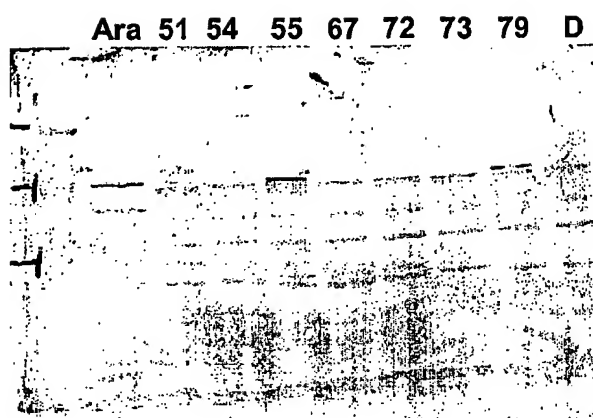


Fig. 7

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25
SEQUENCE LISTING

<110> Bayer CropScience GmbH

<120> Methods for identifying proteins with starch phosphorylating enzymatic activity

<130> BCS 04-5001-PCT

<150> EP04090483.1

<151> 2004-12-15

<150> EP04090121.7

<151> 2004-03-29

<150> EP04090087.0

<151> 2004-03-05

<150> US60/549,980 provisional

<151> 2004-03-05

<160> 26

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Arg Val Asn Leu Ser His Gln Ser His Arg Leu Arg Asn Ser Asn Ser	
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cgt ctc act tgc act gct act tct tct tcc acc att gag gaa caa cgg	192
Arg Leu Thr Cys Thr Ala Thr Ser Ser Ser Thr Ile Glu Glu Gln Arg	
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BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

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His Asp Val Gly Asp Asp Arg Val Val Gly Ser Glu Asn Gly Ala Gln
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Ala Gly Val Leu Leu Arg Arg Gly Ala Met Ala Leu Pro Gly Arg Arg	
30 35 40 45	
ggc ttc gcg tgc cgc ggg aga tcc gcg gcc tcg gcg gca gag aga aca	195
Gly Phe Ala Cys Arg Gly Arg Ser Ala Ala Ser Ala Ala Glu Arg Thr	
50 55 60	
aag gag aaa aag aga aga gat tct tca aag cag cca ttg gtg cat ctc	243
Lys Glu Lys Lys Arg Arg Asp Ser Ser Lys Gln Pro Leu Val His Leu	
65 70 75	
cag gtt tgt cta gag cac cag gtt aag ttt ggt gag cat gta ggc att	291
Gln Val Cys Leu Glu His Gln Val Lys Phe Gly Glu His Val Gly Ile	
80 85 90	
atc ggt tcc aca aag gag ctt ggt tca tgg gag gag cag gtt gaa ctg	339
Ile Gly Ser Thr Lys Glu Leu Gly Ser Trp Glu Glu Gln Val Glu Leu	
95 100 105	
gaa tgg act aca aat ggt tgg gtc tgc cag ctt aag ctc cct gga gaa	387
Glu Trp Thr Thr Asn Gly Trp Val Cys Gln Leu Lys Leu Pro Gly Glu	
110 115 120 125	
aca ctt gtg gag ttt aaa ttt gtt ata ttt ttg gtg gga gga aaa gat	435
Thr Leu Val Glu Phe Lys Phe Val Ile Phe Leu Val Gly Gly Lys Asp	
130 135 140	

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aaa ata tgg gaa gat ggt aat aac cgt gtt gtt gag ctg ccg aag gat	483
Lys Ile Trp Glu Asp Gly Asn Asn Arg Val Val Glu Leu Pro Lys Asp	
145 150 155	
ggt aag ttt gat ata gta tgc cac tgg aat aga aca gaa gag cca tta	531
Gly Lys Phe Asp Ile Val Cys His Trp Asn Arg Thr Glu Glu Pro Leu	
160 165 170	
gaa ctt tta gga aca cca aag ttt gag ttg gtc gga gaa gct gaa aag	579
Glu Leu Leu Gly Thr Pro Lys Phe Glu Leu Val Gly Glu Ala Glu Lys	
175 180 185	
aat act ggc gag gat gct tca gca tct gta act ttt gca cct gaa aaa	627
Asn Thr Gly Glu Asp Ala Ser Ala Ser Val Thr Phe Ala Pro Glu Lys	
190 195 200 205	
gtt caa gat att tca gtt gtt gag aat ggt gat cca gca cca gag gcc	675
Val Gln Asp Ile Ser Val Val Glu Asn Gly Asp Pro Ala Pro Glu Ala	
210 215 220	
gag tca agc aaa ttt ggt ggg caa tgg caa gga agt aaa act gtt ttc	723
Glu Ser Ser Lys Phe Gly Gly Gln Trp Gln Gly Ser Lys Thr Val Phe	
225 230 235	
atg aga tca aat gag cat ctg aat aag gag gct gat agg atg tgg gat	771
Met Arg Ser Asn Glu His Leu Asn Lys Glu Ala Asp Arg Met Trp Asp	
240 245 250	
aca act ggg ctt gat gga ata gca ctg aaa ctg gtg gag ggc gat aaa	819
Thr Thr Gly Leu Asp Gly Ile Ala Leu Lys Leu Val Glu Gly Asp Lys	
255 260 265	
gca tcc agg aac tgg tgg cgg aag tta gag gtt gtt cgc ggg ata ttg	867
Ala Ser Arg Asn Trp Trp Arg Lys Leu Glu Val Val Arg Gly Ile Leu	
270 275 280 285	
tca gaa tct ttt gat gac cag agt cgt ctg ggg gcc ctt gta tac tca	915
Ser Glu Ser Phe Asp Asp Gln Ser Arg Leu Gly Ala Leu Val Tyr Ser	
290 295 300	
gct att tat ctg aag tgg att tat aca ggt cag ata tcg tgc ttt gaa	963
Ala Ile Tyr Leu Lys Trp Ile Tyr Thr Gly Gln Ile Ser Cys Phe Glu	
305 310 315	
gat ggt ggc cac cat cgg cct aac aaa cat gct gag ata tcg agg caa	1011
Asp Gly Gly His His Arg Pro Asn Lys His Ala Glu Ile Ser Arg Gln	
320 325 330	
ata ttc cgt gaa ctt gaa atg atg tat tat ggg aaa acc aca tca gcc	1059
Ile Phe Arg Glu Leu Glu Met Met Tyr Tyr Gly Lys Thr Thr Ser Ala	
335 340 345	
aag gat gtt ctc gtg att cgc aaa att cat ccc ttt tta cct tca ttt	1107
Lys Asp Val Leu Val Ile Arg Lys Ile His Pro Phe Leu Pro Ser Phe	
350 355 360 365	
aag tca gag ttt aca gcc tct gtc cct cta aca cga att cgt gat att	1155
Lys Ser Glu Phe Thr Ala Ser Val Pro Leu Thr Arg Ile Arg Asp Ile	
370 375 380	
gct cac cgg aat gac atc cca cat gat ctc aag caa gaa atc aag cat	1203
Ala His Arg Asn Asp Ile Pro His Asp Leu Lys Gln Glu Ile Lys His	
385 390 395	
act ata caa aac aaa ctt cat cgt aat gct gga cct gag gat ctt att	1251
Thr Ile Gln Asn Lys Leu His Arg Asn Ala Gly Pro Glu Asp Leu Ile	
400 405 410	

BCS 04-501-PCT_SEQUENZPROTOKOLL-Verfahren zur Identifizierung.ST25																1299
gct Ala	aca Thr	gaa Glu	gtc Val	atg Met	ctt Leu	gct Ala	agg Arg	att Ile	act Thr	aag Lys	acc Thr	cct Pro	gga Gly	gaa Glu	tac Tyr	
415						420					425					
agt Ser	gaa Glu	aca Thr	ttt Phe	gtt Val	gaa Glu	caa Gln	ttc Phe	acg Thr	ata Ile	ttt Phe	tat Tyr	agc Ser	gaa Glu	cta Leu	aaa Lys	1347
430					435					440					445	
gat Asp	ttc Phe	ttc Phe	aat Asn	gct Ala	ggc Gly	agc Ser	cta Leu	ttt Phe	gag Glu	caa Gln	ctg Leu	gag Glu	tcc Ser	atc Ile	aag Lys	1395
				450					455					460		
gaa Glu	tct Ser	ctg Leu	aac Asn	gag Glu	tca Ser	ggc Gly	tta Leu	gaa Glu	gtt Val	ctc Leu	tca Ser	tcc Ser	ttt Phe	gtg Val	gaa Glu	1443
			465					470					475			
acc Thr	aaa Lys	agg Arg	agt Ser	ttg Leu	gac Asp	caa Gln	gtg Val	gat Asp	cat His	gca Ala	gaa Glu	gat Asp	ttg Leu	gat Asp	aaa Lys	1491
		480				485						490				
aat Asn	gat Asp	acc Thr	att Ile	caa Gln	att Ile	ttg Leu	atg Met	act Thr	acc Thr	ttg Leu	caa Gln	tca Ser	tta Leu	tct Ser	tct Ser	1539
	495					500					505					
cta Leu	aga Arg	tcg Ser	gtt Val	cta Leu	atg Met	aag Lys	ggc Gly	ctt Leu	gaa Glu	agt Ser	ggc Gly	ctt Leu	aga Arg	aat Asn	gat Asp	1587
510					515					520					525	
gcg Ala	cct Pro	gat Asp	aat Asn	gct Ala	ata Ile	gca Ala	atg Met	cga Arg	caa Gln	aag Lys	tgg Trp	cgc Arg	ctt Leu	tgt Cys	gaa Glu	1635
				530					535					540		
att Ile	agt Ser	ctt Leu	gag Glu	gat Asp	tat Tyr	tca Ser	ttt Phe	gtt Val	ctg Leu	tta Leu	agc Ser	aga Arg	ttc Phe	atc Ile	aat Asn	1683
			545					550					555			
act Thr	ctt Leu	gaa Glu	gcc Ala	tta Leu	ggt Gly	gga Gly	tca Ser	gct Ala	tca Ser	ctt Leu	gca Ala	aag Lys	gat Asp	gta Val	gct Ala	1731
		560				565						570				
aga Arg	aat Asn	act Thr	act Thr	cta Leu	tgg Trp	gat Asp	act Thr	act Thr	ctt Leu	gat Asp	gcc Ala	ctt Leu	gtc Val	att Ile	ggc Gly	1779
	575					580					585					
atc Ile	aat Asn	caa Gln	gtt Val	agc Ser	ttt Phe	tca Ser	ggt Gly	tgg Trp	aaa Lys	aca Thr	gat Asp	gaa Glu	tgt Cys	att Ile	gcc Ala	1827
590					595					600					605	
ata Ile	ggg Gly	aat Asn	gag Glu	att Ile	ctt Leu	tcc Ser	tgg Trp	aag Lys	caa Gln	aaa Lys	ggt Gly	cta Leu	tct Ser	gaa Glu	agt Ser	1875
				610					615					620		
gaa Glu	ggt Gly	tgt Cys	gaa Glu	gat Asp	ggg Gly	aaa Lys	tat Tyr	att Ile	tgg Trp	tca Ser	cta Leu	aga Arg	ctt Leu	aaa Lys	gct Ala	1923
			625					630					635			
aca Thr	ctg Leu	gac Asp	aga Arg	gca Ala	cgg Arg	aga Arg	tta Leu	acg Thr	gaa Glu	gag Glu	tac Tyr	tct Ser	gaa Glu	gca Ala	ctt Leu	1971
		640					645					650				
ctt Leu	tct Ser	ata Ile	ttc Phe	cct Pro	gaa Glu	aaa Lys	gta Val	atg Met	gtt Val	att Ile	ggg Gly	aaa Lys	gcc Ala	ctt Leu	gga Gly	2019
	655					660					665					
ata Ile	cca Pro	gat Asp	aac Asn	agt Ser	gtg Val	aga Arg	act Thr	tac Tyr	aca Thr	gag Glu	gca Ala	gaa Glu	att Ile	cgt Arg	gct Ala	2067
670					675					680					685	

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ggc att gtt ttt cag gta tct aaa cta tgc aca gta ctt cag aaa gca															2115	
Gly Ile Val Phe Gln Val Ser Lys Leu Cys Thr Val Leu Gln Lys Ala																
			690					695					700			
att cga gaa gta ctt gga tca act ggc tgg gat gtt ctt gtt cct gga															2163	
Ile Arg Glu Val Leu Gly Ser Thr Gly Trp Asp Val Leu Val Pro Gly																
			705					710					715			
gtg gcc cat gga act ctg atg cgg gtg gaa aga att ctt cct gga tca															2211	
Val Ala His Gly Thr Leu Met Arg Val Glu Arg Ile Leu Pro Gly Ser																
			720					725					730			
tta cct tca tct gtc aaa gaa cct gtg gtt cta att gta gat aag gct															2259	
Leu Pro Ser Ser Val Lys Glu Pro Val Val Leu Ile Val Asp Lys Ala																
			735					740					745			
gat gga gat gaa gag gtc aaa gct gct ggg gat aat ata gtt ggt gtt															2307	
Asp Gly Asp Glu Glu Val Lys Ala Ala Gly Asp Asn Ile Val Gly Val																
			750					755					760			
att ctt ctt cag gaa cta cct cac ctt tca cat ctt ggt gtt aga gct															2355	
Ile Leu Leu Gln Glu Leu Pro His Leu Ser His Leu Gly Val Arg Ala																
			770					775					780			
cgt caa gag aat gtt gta ttt gta act tgt gaa tat gat gac aca gtt															2403	
Arg Gln Glu Asn Val Val Phe Val Thr Cys Glu Tyr Asp Asp Thr Val																
			785					790					795			
aca gat gtg tat ttg ctt gag gga aaa tat atc aga tta gaa gca tca															2451	
Thr Asp Val Tyr Leu Leu Glu Gly Lys Tyr Ile Arg Leu Glu Ala Ser																
			800					805					810			
tcc atc aat gtc aat ctc tca ata gtt tca gaa aaa aat gac aat gct															2499	
Ser Ile Asn Val Asn Leu Ser Ile Val Ser Glu Lys Asn Asp Asn Ala																
			815					820					825			
gtc tct aca gaa cca aat agt aca ggg aat cca ttt caa cag aaa ctc															2547	
Val Ser Thr Glu Pro Asn Ser Thr Gly Asn Pro Phe Gln Gln Lys Leu																
			830					835					840			
caa aat gaa ttc tct cta cca tcg gat atc gag atg cca ctg caa atg															2595	
Gln Asn Glu Phe Ser Leu Pro Ser Asp Ile Glu Met Pro Leu Gln Met																
			850					855					860			
tct aag caa aaa agc aaa tca gga gtg aat ggt agt ttt gct gct ctt															2643	
Ser Lys Gln Lys Ser Lys Ser Gly Val Asn Gly Ser Phe Ala Ala Leu																
			865					870					875			
gag ctt tca gaa gct tca gtg gaa tca gct ggt gca aaa gct gct gca															2691	
Glu Leu Ser Glu Ala Ser Val Glu Ser Ala Gly Ala Lys Ala Ala Ala																
			880					885					890			
tgc aga act ctt tct gtt ctt gct tca ttg tct aat aaa gtc tat agt															2739	
Cys Arg Thr Leu Ser Val Leu Ala Ser Leu Ser Asn Lys Val Tyr Ser																
			895					900					905			
gat caa gga gtt cca gca gcc ttt aga gtc cct tct ggt gct gtg ata															2787	
Asp Gln Gly Val Pro Ala Ala Phe Arg Val Pro Ser Gly Ala Val Ile																
			910					915					920			
cca ttt gga tca atg gag gat gcg ctc aag aaa agt gga tca ctg gaa															2835	
Pro Phe Gly Ser Met Glu Asp Ala Leu Lys Lys Ser Gly Ser Leu Glu																
			930					935					940			
tcc ttt aca agc ctt cta gaa aag att gaa aca gcc aaa gtc gaa aat															2883	
Ser Phe Thr Ser Leu Leu Glu Lys Ile Glu Thr Ala Lys Val Glu Asn																
			945					950					955			

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ggt gaa gtt gat agc ctg gcg ttg gag cta caa gca ata att tca cat	2931														
Gly Glu Val Asp Ser Leu Ala Leu Glu Leu Gln Ala Ile Ile Ser His															
960	965														
970															
ctt tcc cca ccg gag gag act att ata ttt ctc aaa aga atc ttc cca	2979														
Leu Ser Pro Pro Glu Glu Thr Ile Ile Phe Leu Lys Arg Ile Phe Pro															
975	980														
985															
cag gat gtc cgg ttg att gtt aga tct agt gct aat gtg gag gat ttg	3027														
Gln Asp Val Arg Leu Ile Val Arg Ser Ser Ala Asn Val Glu Asp Leu															
990	995														
1000	1005														
gct ggt atg tca gct gct ggt ctc tat gat tca att ccc aat gtc	3072														
Ala Gly Met Ser Ala Ala Gly Leu Tyr Asp Ser Ile Pro Asn Val															
1010	1015														
1020															
agt ctc atg gac cca tgt gcc ttt gga gct gcg gtt ggg aag gtt	3117														
Ser Leu Met Asp Pro Cys Ala Phe Gly Ala Ala Val Gly Lys Val															
1025	1030														
1035															
tgg gct tct tta tac aca agg aga gcc atc cta agc cgt cga gcc	3162														
Trp Ala Ser Leu Tyr Thr Arg Arg Ala Ile Leu Ser Arg Arg Ala															
1040	1045														
1050															
gct ggt gtt tat cag aga gac gcg aca atg gct gtt ctt gtc caa	3207														
Ala Gly Val Tyr Gln Arg Asp Ala Thr Met Ala Val Leu Val Gln															
1055	1060														
1065															
gaa ata ctg cag cca gat ctc tcc ttc gtg ctt cat act gtt tgc	3252														
Glu Ile Leu Gln Pro Asp Leu Ser Phe Val Leu His Thr Val Cys															
1070	1075														
1080															
ccc gct gac cat gac ccc aag gtt gtc cag gct gag gtc gcc cct	3297														
Pro Ala Asp His Asp Pro Lys Val Val Gln Ala Glu Val Ala Pro															
1085	1090														
1095															
ggg ctg ggt gaa acg ctt gct tca gga acc cgt ggc acc ccg tgg	3342														
Gly Leu Gly Glu Thr Leu Ala Ser Gly Thr Arg Gly Thr Pro Trp															
1100	1105														
1110															
agg ctg tca tgt aac aaa ttc gat gga aaa gtt gcc act ctt gcc	3387														
Arg Leu Ser Cys Asn Lys Phe Asp Gly Lys Val Ala Thr Leu Ala															
1115	1120														
1125															
ttt tca aat ttc agt gag gag atg gtg gtg cac aac tct ggt cct	3432														
Phe Ser Asn Phe Ser Glu Glu Met Val Val His Asn Ser Gly Pro															
1130	1135														
1140															
gcc aat gga gaa gta att cgt ctt act gtt gat tac agc aag aag	3477														
Ala Asn Gly Glu Val Ile Arg Leu Thr Val Asp Tyr Ser Lys Lys															
1145	1150														
1155															
cca ttg tcg gtt gat aca acc ttt agg aag cag ttt ggt cag cga	3522														
Pro Leu Ser Val Asp Thr Thr Phe Arg Lys Gln Phe Gly Gln Arg															
1160	1165														
1170															
ctg gct gcg att ggc cag tat ctg gag cag aag ttc ggg agt gca	3567														
Leu Ala Ala Ile Gly Gln Tyr Leu Glu Gln Lys Phe Gly Ser Ala															
1175	1180														
1185															
cag gat gtg gaa ggt tgc ctg gtt ggg aaa gat att ttt ata gtg	3612														
Gln Asp Val Glu Gly Cys Leu Val Gly Lys Asp Ile Phe Ile Val															
1190	1195														
1200															
caa agc agg cca cag cca tag aagccgaatt c	3644														
Gln Ser Arg Pro Gln Pro															
1205															

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<210> 4

<211> 1206

<212> PRT

<213> Oryza sativa

<400> 4

Met Thr Ser Leu Arg Pro Leu Glu Thr Ser Leu Ser Ile Gly Gly Arg
 1 5 10 15

Pro Arg Arg Gly Leu Val Leu Pro Pro Pro Gly Val Gly Ala Gly Val
 20 25 30

Leu Leu Arg Arg Gly Ala Met Ala Leu Pro Gly Arg Arg Gly Phe Ala
 35 40 45

Cys Arg Gly Arg Ser Ala Ala Ser Ala Ala Glu Arg Thr Lys Glu Lys
 50 55 60

Lys Arg Arg Asp Ser Ser Lys Gln Pro Leu Val His Leu Gln Val Cys
 65 70 75 80

Leu Glu His Gln Val Lys Phe Gly Glu His Val Gly Ile Ile Gly Ser
 85 90 95

Thr Lys Glu Leu Gly Ser Trp Glu Glu Gln Val Glu Leu Glu Trp Thr
 100 105 110

Thr Asn Gly Trp Val Cys Gln Leu Lys Leu Pro Gly Glu Thr Leu Val
 115 120 125

Glu Phe Lys Phe Val Ile Phe Leu Val Gly Gly Lys Asp Lys Ile Trp
 130 135 140

Glu Asp Gly Asn Asn Arg Val Val Glu Leu Pro Lys Asp Gly Lys Phe
 145 150 155 160

Asp Ile Val Cys His Trp Asn Arg Thr Glu Glu Pro Leu Glu Leu Leu
 165 170 175

Gly Thr Pro Lys Phe Glu Leu Val Gly Glu Ala Glu Lys Asn Thr Gly
 180 185 190

Glu Asp Ala Ser Ala Ser Val Thr Phe Ala Pro Glu Lys Val Gln Asp
 195 200 205

Ile Ser Val Val Glu Asn Gly Asp Pro Ala Pro Glu Ala Glu Ser Ser
 210 215 220

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Lys Phe Gly Gly Gln Trp Gln Gly Ser Lys Thr Val Phe Met Arg Ser
 225 230 235 240

Asn Glu His Leu Asn Lys Glu Ala Asp Arg Met Trp Asp Thr Thr Gly
 245 250 255

Leu Asp Gly Ile Ala Leu Lys Leu Val Glu Gly Asp Lys Ala Ser Arg
 260 265 270

Asn Trp Trp Arg Lys Leu Glu Val Val Arg Gly Ile Leu Ser Glu Ser
 275 280 285

Phe Asp Asp Gln Ser Arg Leu Gly Ala Leu Val Tyr Ser Ala Ile Tyr
 290 295 300

Leu Lys Trp Ile Tyr Thr Gly Gln Ile Ser Cys Phe Glu Asp Gly Gly
 305 310 315 320

His His Arg Pro Asn Lys His Ala Glu Ile Ser Arg Gln Ile Phe Arg
 325 330 335

Glu Leu Glu Met Met Tyr Tyr Gly Lys Thr Thr Ser Ala Lys Asp Val
 340 345 350

Leu Val Ile Arg Lys Ile His Pro Phe Leu Pro Ser Phe Lys Ser Glu
 355 360 365

Phe Thr Ala Ser Val Pro Leu Thr Arg Ile Arg Asp Ile Ala His Arg
 370 375 380

Asn Asp Ile Pro His Asp Leu Lys Gln Glu Ile Lys His Thr Ile Gln
 385 390 395 400

Asn Lys Leu His Arg Asn Ala Gly Pro Glu Asp Leu Ile Ala Thr Glu
 405 410 415

Val Met Leu Ala Arg Ile Thr Lys Thr Pro Gly Glu Tyr Ser Glu Thr
 420 425 430

Phe Val Glu Gln Phe Thr Ile Phe Tyr Ser Glu Leu Lys Asp Phe Phe
 435 440 445

Asn Ala Gly Ser Leu Phe Glu Gln Leu Glu Ser Ile Lys Glu Ser Leu
 450 455 460

Asn Glu Ser Gly Leu Glu Val Leu Ser Ser Phe Val Glu Thr Lys Arg
 465 470 475 480

Ser Leu Asp Gln Val Asp His Ala Glu Asp Leu Asp Lys Asn Asp Thr
 485 490 495

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Ile Gln Ile Leu Met Thr Thr Leu Gln Ser Leu Ser Ser Leu Arg Ser
 500 505 510

Val Leu Met Lys Gly Leu Glu Ser Gly Leu Arg Asn Asp Ala Pro Asp
 515 520 525

Asn Ala Ile Ala Met Arg Gln Lys Trp Arg Leu Cys Glu Ile Ser Leu
 530 535 540

Glu Asp Tyr Ser Phe Val Leu Leu Ser Arg Phe Ile Asn Thr Leu Glu
 545 550 555 560

Ala Leu Gly Gly Ser Ala Ser Leu Ala Lys Asp Val Ala Arg Asn Thr
 565 570 575

Thr Leu Trp Asp Thr Thr Leu Asp Ala Leu Val Ile Gly Ile Asn Gln
 580 585 590

Val Ser Phe Ser Gly Trp Lys Thr Asp Glu Cys Ile Ala Ile Gly Asn
 595 600 605

Glu Ile Leu Ser Trp Lys Gln Lys Gly Leu Ser Glu Ser Glu Gly Cys
 610 615 620

Glu Asp Gly Lys Tyr Ile Trp Ser Leu Arg Leu Lys Ala Thr Leu Asp
 625 630 635 640

Arg Ala Arg Arg Leu Thr Glu Glu Tyr Ser Glu Ala Leu Leu Ser Ile
 645 650 655

Phe Pro Glu Lys Val Met Val Ile Gly Lys Ala Leu Gly Ile Pro Asp
 660 665 670

Asn Ser Val Arg Thr Tyr Thr Glu Ala Glu Ile Arg Ala Gly Ile Val
 675 680 685

Phe Gln Val Ser Lys Leu Cys Thr Val Leu Gln Lys Ala Ile Arg Glu
 690 695 700

Val Leu Gly Ser Thr Gly Trp Asp Val Leu Val Pro Gly Val Ala His
 705 710 715 720

Gly Thr Leu Met Arg Val Glu Arg Ile Leu Pro Gly Ser Leu Pro Ser
 725 730 735

Ser Val Lys Glu Pro Val Val Leu Ile Val Asp Lys Ala Asp Gly Asp
 740 745 750

Glu Glu Val Lys Ala Ala Gly Asp Asn Ile Val Gly Val Ile Leu Leu
 755 760 765

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Gln Glu Leu Pro His Leu Ser His Leu Gly Val Arg Ala Arg Gln Glu
 770 775 780
 Asn Val Val Phe Val Thr Cys Glu Tyr Asp Asp Thr Val Thr Asp Val
 785 790 795 800
 Tyr Leu Leu Glu Gly Lys Tyr Ile Arg Leu Glu Ala Ser Ser Ile Asn
 805 810 815
 Val Asn Leu Ser Ile Val Ser Glu Lys Asn Asp Asn Ala Val Ser Thr
 820 825 830
 Glu Pro Asn Ser Thr Gly Asn Pro Phe Gln Gln Lys Leu Gln Asn Glu
 835 840 845
 Phe Ser Leu Pro Ser Asp Ile Glu Met Pro Leu Gln Met Ser Lys Gln
 850 855 860
 Lys Ser Lys Ser Gly Val Asn Gly Ser Phe Ala Ala Leu Glu Leu Ser
 865 870 875 880
 Glu Ala Ser Val Glu Ser Ala Gly Ala Lys Ala Ala Ala Cys Arg Thr
 885 890 895
 Leu Ser Val Leu Ala Ser Leu Ser Asn Lys Val Tyr Ser Asp Gln Gly
 900 905 910
 Val Pro Ala Ala Phe Arg Val Pro Ser Gly Ala Val Ile Pro Phe Gly
 915 920 925
 Ser Met Glu Asp Ala Leu Lys Lys Ser Gly Ser Leu Glu Ser Phe Thr
 930 935 940
 Ser Leu Leu Glu Lys Ile Glu Thr Ala Lys Val Glu Asn Gly Glu Val
 945 950 955 960
 Asp Ser Leu Ala Leu Glu Leu Gln Ala Ile Ile Ser His Leu Ser Pro
 965 970 975
 Pro Glu Glu Thr Ile Ile Phe Leu Lys Arg Ile Phe Pro Gln Asp Val
 980 985 990
 Arg Leu Ile Val Arg Ser Ser Ala Asn Val Glu Asp Leu Ala Gly Met
 995 1000 1005
 Ser Ala Ala Gly Leu Tyr Asp Ser Ile Pro Asn Val Ser Leu Met
 1010 1015 1020
 Asp Pro Cys Ala Phe Gly Ala Ala Val Gly Lys Val Trp Ala Ser
 1025 1030 1035

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Leu Tyr Thr Arg Arg Ala Ile Leu Ser Arg Arg Ala Ala Gly Val
 1040 1045 1050
 Tyr Gln Arg Asp Ala Thr Met Ala Val Leu Val Gln Glu Ile Leu
 1055 1060 1065
 Gln Pro Asp Leu Ser Phe Val Leu His Thr Val Cys Pro Ala Asp
 1070 1075 1080
 His Asp Pro Lys Val Val Gln Ala Glu Val Ala Pro Gly Leu Gly
 1085 1090 1095
 Glu Thr Leu Ala Ser Gly Thr Arg Gly Thr Pro Trp Arg Leu Ser
 1100 1105 1110
 Cys Asn Lys Phe Asp Gly Lys Val Ala Thr Leu Ala Phe Ser Asn
 1115 1120 1125
 Phe Ser Glu Glu Met Val Val His Asn Ser Gly Pro Ala Asn Gly
 1130 1135 1140
 Glu Val Ile Arg Leu Thr Val Asp Tyr Ser Lys Lys Pro Leu Ser
 1145 1150 1155
 Val Asp Thr Thr Phe Arg Lys Gln Phe Gly Gln Arg Leu Ala Ala
 1160 1165 1170
 Ile Gly Gln Tyr Leu Glu Gln Lys Phe Gly Ser Ala Gln Asp Val
 1175 1180 1185
 Glu Gly Cys Leu Val Gly Lys Asp Ile Phe Ile Val Gln Ser Arg
 1190 1195 1200
 Pro Gln Pro
 1205

<210> 5

<211> 12

<212> PRT

<213> Oryza sativa, Arabidopsis thaliana, sorghum bicolor

<400> 5

Leu Pro His Leu Ser His Leu Gly Val Arg Ala Arg
 1 5 10

<210> 6

<211> 7

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<212> PRT

<213> Hordeum vulgare

<400> 6

Ser Arg Arg Val Ala Gly Val
1 5

<210> 7

<211> 7

<212> PRT

<213> Hordeum vulgare

<400> 7

Val Glu Ala Glu Val Ala Pro
1 5

<210> 8

<211> 9

<212> PRT

<213> Hordeum vulgare

<400> 8

His Thr Val Ser Pro Ser Asp His Asp
1 5

<210> 9

<211> 807

<212> DNA

<213> Hordeum vulgare

<220>

<221> CDS

<222> (3)..(590)

<223>

<400> 9

cg gca cga gga gtc ctc ccc aat gtg agc ctc tcg gac cca acc aac
Ala Arg Gly Val Leu Pro Asn Val Ser Leu Ser Asp Pro Thr Asn

47

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25																	
1	5				10				15								
ttc ggg tct gca gta gcg cgg gtc tgg gcc tcg ctg tac act cgg agg	Phe Gly Ser Ala Val Ala Arg Val Trp Ala Ser Leu Tyr Thr Arg Arg				20 25 30												95
gcc atc ctc agc cgc cgg gtg gct ggc gtg ccc cag agg gac gcc aag	Ala Ile Leu Ser Arg Arg Val Ala Gly Val Pro Gln Arg Asp Ala Lys				35 40 45												143
atg gct gtc ctg gtg cag gag atg ctg gag cca gag cta tcc ttc gtg	Met Ala Val Leu Val Gln Glu Met Leu Glu Pro Glu Leu Ser Phe Val				50 55 60												191
ctc cac acg gtc agc ccc tcg gac cac gac acc agg gtc gtc gag gct	Leu His Thr Val Ser Pro Ser Asp His Asp Thr Arg Val Val Glu Ala				65 70 75												239
gag gtt gcc ccg ggg ctg ggc gag acc ctt gcc gct ggc acc cgc ggc	Glu Val Ala Pro Gly Leu Gly Glu Thr Leu Ala Ala Gly Thr Arg Gly				80 85 90 95												287
acc ccg tgg cgt ctc tcc tgc gac aag ttc gac acc gac gtc gcc acc	Thr Pro Trp Arg Leu Ser Cys Asp Lys Phe Asp Thr Asp Val Ala Thr				100 105 110												335
ctg gcc ttc gcc aac ttc agt gag gag atg cgg gtg ctc ggc tcg ggc	Leu Ala Phe Ala Asn Phe Ser Glu Glu Met Arg Val Leu Gly Ser Gly				115 120 125												383
ccc gcc gac ggc gag gtg gtg agg ctc act gtc gac tac agc acg aag	Pro Ala Asp Gly Glu Val Val Arg Leu Thr Val Asp Tyr Ser Thr Lys				130 135 140												431
ctg ctc tcc gtc gac agg acc ttc agg cag aag ttc ggt cag cgg ctg	Leu Leu Ser Val Asp Arg Thr Phe Arg Gln Lys Phe Gly Gln Arg Leu				145 150 155												479
gcc gcc gtg ggg cag tac ctg gag cag agg ttc ggg agc gcc cag gac	Ala Ala Val Gly Gln Tyr Leu Glu Gln Arg Phe Gly Ser Ala Gln Asp				160 165 170 175												527
gtg gag ggc tgc atg gtc tgg gaa gac atc tac ata gtg cag agc atg	Val Glu Gly Cys Met Val Trp Glu Asp Ile Tyr Ile Val Gln Ser Met				180 185 190												575
cca caa ccg ctg tag agtcatccgt aataatgttt agatgagcaa agtttttggt	Pro Gln Pro Leu				195												630
ggtgaaataa aatttgccga aaatcccatg gcaaaataag tcaggtatga agagcccgcc																690	
tgcgaaacca actgattcta aataatgttt tgaattcgtg tttaaattat gggacgtgaa																750	
caatgatttc cttggaatgc atgcattgta agttttaaaa aaaaaaaaaa aaaaaaa																807	

<210> 10

<211> 195

<212> PRT

<213> Hordeum vulgare

<400> 10

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

Ala Arg Gly Val Leu Pro Asn Val Ser Leu Ser Asp Pro Thr Asn Phe
 1 5 10 15

Gly Ser Ala Val Ala Arg Val Trp Ala Ser Leu Tyr Thr Arg Arg Ala
 20 25 30

Ile Leu Ser Arg Arg Val Ala Gly Val Pro Gln Arg Asp Ala Lys Met
 35 40 45

Ala Val Leu Val Gln Glu Met Leu Glu Pro Glu Leu Ser Phe Val Leu
 50 55 60

His Thr Val Ser Pro Ser Asp His Asp Thr Arg Val Val Glu Ala Glu
 65 70 75 80

Val Ala Pro Gly Leu Gly Glu Thr Leu Ala Ala Gly Thr Arg Gly Thr
 85 90 95

Pro Trp Arg Leu Ser Cys Asp Lys Phe Asp Thr Asp Val Ala Thr Leu
 100 105 110

Ala Phe Ala Asn Phe Ser Glu Glu Met Arg Val Leu Gly Ser Gly Pro
 115 120 125

Ala Asp Gly Glu Val Val Arg Leu Thr Val Asp Tyr Ser Thr Lys Leu
 130 135 140

Leu Ser Val Asp Arg Thr Phe Arg Gln Lys Phe Gly Gln Arg Leu Ala
 145 150 155 160

Ala Val Gly Gln Tyr Leu Glu Gln Arg Phe Gly Ser Ala Gln Asp Val
 165 170 175

Glu Gly Cys Met Val Trp Glu Asp Ile Tyr Ile Val Gln Ser Met Pro
 180 185 190

Gln Pro Leu
 195

<210> 11

<211> 9

<212> PRT

<213> Solanum tuberosum

<400> 11

Pro Glu Glu Cys Lys Ala Val Gly Asn
 1 5

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

<210> 12

<211> 7

<212> PRT

<213> Solanum tuberosum

<400> 12

Thr Glu Glu Tyr Ser Glu Thr
1 5

<210> 13

<211> 7

<212> PRT •

<213> Solanum tuberosum

<400> 13

Arg Phe Val Asn Ala Val Glu
1 5

<210> 14

<211> 7

<212> PRT

<213> Solanum tuberosum

<400> 14

Glu Gly Ser Glu Asp Gly Lys
1 5

<210> 15

<211> 403

<212> DNA

<213> Solanum tuberosum

<220>

<221> CDS

<222> (1)..(402)

<223>

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

<400> 15
 gcg gat gct tca ata gct atg cgt cag aag tgg cgt ctc tgc gaa atc 48
 Ala Asp Ala Ser Ile Ala Met Arg Gln Lys Trp Arg Leu Cys Glu Ile
 1 5 10 15

ggg ctt gaa gac tat gca ttt gtt ctt ttg agc agg ttt gtg aat gca 96
 Gly Leu Glu Asp Tyr Ala Phe Val Leu Ser Arg Phe Val Asn Ala
 20 25 30

ggt gaa gct cta ggc gga gct gat tgg ctt gca gag aat gta aca gtg 144
 Val Glu Ala Leu Gly Gly Ala Asp Trp Leu Ala Glu Asn Val Thr Val
 35 40 45

aaa aac att agt tct tgg aat gat cca att gga gca ctt aca gtt gga 192
 Lys Asn Ile Ser Ser Trp Asn Asp Pro Ile Gly Ala Leu Thr Val Gly
 50 55 60

atc caa cag cta ggt ata tct ggt tgg aag ccc gag gaa tgc aaa gct 240
 Ile Gln Gln Leu Gly Ile Ser Gly Trp Lys Pro Glu Glu Cys Lys Ala
 65 70 75 80

ggt gga aat gaa ctt ttg tca tgg aaa gaa agg ggt att tca gaa att 288
 Val Gly Asn Glu Leu Leu Ser Trp Lys Glu Arg Gly Ile Ser Glu Ile
 85 90 95

gaa ggc agc gaa gat ggt aag act ata tgg gca tta aga cta aaa gcg 336
 Glu Gly Ser Glu Asp Gly Lys Thr Ile Trp Ala Leu Arg Leu Lys Ala
 100 105 110

act ctt gat aga agt cga agg tta act gag gag tat tcc gag aca ctt 384
 Thr Leu Asp Arg Ser Arg Arg Leu Thr Glu Glu Tyr Ser Glu Thr Leu
 115 120 125

ctc caa ata ttc cct gaa a 403
 Leu Gln Ile Phe Pro Glu
 130

<210> 16

<211> 134

<212> PRT

<213> solanum tuberosum

<400> 16

Ala Asp Ala Ser Ile Ala Met Arg Gln Lys Trp Arg Leu Cys Glu Ile
 1 5 10 15

Gly Leu Glu Asp Tyr Ala Phe Val Leu Leu Ser Arg Phe Val Asn Ala
 20 25 30

Val Glu Ala Leu Gly Gly Ala Asp Trp Leu Ala Glu Asn Val Thr Val
 35 40 45

Lys Asn Ile Ser Ser Trp Asn Asp Pro Ile Gly Ala Leu Thr Val Gly
 50 55 60

Ile Gln Gln Leu Gly Ile Ser Gly Trp Lys Pro Glu Glu Cys Lys Ala

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25
 65 70 75 80

Val Gly Asn Glu Leu Leu Ser Trp Lys Glu Arg Gly Ile Ser Glu Ile
 85 90 95

Glu Gly Ser Glu Asp Gly Lys Thr Ile Trp Ala Leu Arg Leu Lys Ala
 100 105 110

Thr Leu Asp Arg Ser Arg Arg Leu Thr Glu Glu Tyr Ser Glu Thr Leu
 115 120 125

Leu Gln Ile Phe Pro Glu
 130

<210> 17

<211> 7

<212> PRT

<213> sorghum bicolor

<400> 17

Asp Gly Gly His His Arg Pro
 1 5

<210> 18

<211> 8

<212> PRT

<213> sorghum bicolor

<400> 18

Asp Ala Pro Asp Ser Ala Ile Ala
 1 5

<210> 19

<211> 9

<212> PRT

<213> sorghum bicolor

<400> 19

Ile Pro Glu Asn Ser Val Arg Thr Tyr
 1 5

<210> 20

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

<211> 6

<212> PRT

<213> Sorghum bicolor

<400> 20

Val Asn Lys Ala Asp Gly
 1 5

<210> 21

<211> 1526

<212> DNA

<213> Sorghum bicolor

<220>

<221> CDS

<222> (2)..(1525)

<223>

<400> 21

g cac gag gct gaa tat gtt cat gat cag agt cac ctg gag gct ctt aca	49
His Glu Ala Glu Tyr Val His Asp Gln Ser His Leu Glu Ala Leu Thr	
1 5 10 15	
tat tct gca ata tat cta aag tgg ata tat act ggt caa ata cca tgc	97
Tyr Ser Ala Ile Tyr Leu Lys Trp Ile Tyr Thr Gly Gln Ile Pro Cys	
20 25 30	
ttt gag gat ggt ggt cac cat cga ccc aat aaa cat gct gag ata tcc	145
Phe Glu Asp Gly Gly His His Arg Pro Asn Lys His Ala Glu Ile Ser	
35 40 45	
agg caa att ttt cgt gaa att gaa agg ata tac tat ggg gaa aac aca	193
Arg Gln Ile Phe Arg Glu Ile Glu Arg Ile Tyr Tyr Gly Glu Asn Thr	
50 55 60	
tca gct cag gat ttg ctt gtg ata cgc aag att cat cct tgt cta cct	241
Ser Ala Gln Asp Leu Leu Val Ile Arg Lys Ile His Pro Cys Leu Pro	
65 70 75 80	
tca ttt aaa tca gaa ttt act gcc tct gtt cct cta aca cga att cgt	289
Ser Phe Lys Ser Glu Phe Thr Ala Ser Val Pro Leu Thr Arg Ile Arg	
85 90 95	
gat att gct cat cgt aat gac ata cca cat gat ctc aag caa gaa atc	337
Asp Ile Ala His Arg Asn Asp Ile Pro His Asp Leu Lys Gln Glu Ile	
100 105 110	
aag cat act ata caa aac aag ctt cac cgg aat gcc ggc cct gag gat	385
Lys His Thr Ile Gln Asn Lys Leu His Arg Asn Ala Gly Pro Glu Asp	
115 120 125	

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BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25
 cgt gct ggt gtt att ttt cac gtc tcg aaa ctt tgc act gta ctt tta 1249
 Arg Ala Gly Val Ile Phe His Val Ser Lys Leu Cys Thr Val Leu Leu
 405 410 415
 aaa gca act cga gca gtt ctt gga tcg tct gtg tgg gat gtt ctt gtt 1297
 Lys Ala Thr Arg Ala Val Leu Gly Ser Ser Val Trp Asp Val Leu Val
 420 425 430
 cct gga gtg gcc cat gga gcc ttg ata cag gtt gaa aga ata gct cct 1345
 Pro Gly Val Ala His Gly Ala Leu Ile Gln Val Glu Arg Ile Ala Pro
 435 440 445
 gga tca ttg cca tca tcc atc aaa gaa cct gtc gtg cta gtt gta aac 1393
 Gly Ser Leu Pro Ser Ser Ile Lys Glu Pro Val Val Leu Val Val Asn
 450 455 460
 aag gct gat gga gat gaa gag gtc aaa gct gct ggg gat aac ata gtg 1441
 Lys Ala Asp Gly Asp Glu Glu Val Lys Ala Ala Gly Asp Asn Ile Val
 465 470 475 480
 ggt gtt att ctt cta caa gaa tta cct cac cta tca cat ctt ggt gtt 1489
 Gly Val Ile Leu Leu Gln Glu Leu Pro His Leu Ser His Leu Gly Val
 485 490 495
 aga gct cgt caa gag aaa gtt gta ttt gta act tgc g 1526
 Arg Ala Arg Gln Glu Lys Val Val Phe Val Thr Cys
 500 505

<210> 22

<211> 508

<212> PRT

<213> sorghum bicolor

<400> 22

His Glu Ala Glu Tyr Val His Asp Gln Ser His Leu Glu Ala Leu Thr
 1 5 10 15
 Tyr Ser Ala Ile Tyr Leu Lys Trp Ile Tyr Thr Gly Gln Ile Pro Cys
 20 25 30
 Phe Glu Asp Gly Gly His His Arg Pro Asn Lys His Ala Glu Ile Ser
 35 40 45
 Arg Gln Ile Phe Arg Glu Ile Glu Arg Ile Tyr Tyr Gly Glu Asn Thr
 50 55 60
 Ser Ala Gln Asp Leu Leu Val Ile Arg Lys Ile His Pro Cys Leu Pro
 65 70 75 80
 Ser Phe Lys Ser Glu Phe Thr Ala Ser Val Pro Leu Thr Arg Ile Arg
 85 90 95
 Asp Ile Ala His Arg Asn Asp Ile Pro His Asp Leu Lys Gln Glu Ile
 100 105 110

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

Lys His Thr Ile Gln Asn Lys Leu His Arg Asn Ala Gly Pro Glu Asp
 115 120 125
 Leu Ile Ala Thr Glu Ala Met Leu Ala Arg Ile Thr Lys Thr Pro Gly
 130 135 140
 Glu Tyr Ser Glu Ala Phe Val Glu Gln Phe Lys Thr Phe Tyr Ser Glu
 145 150 155 160
 Leu Lys Asp Phe Phe Asn Ala Gly Ser Leu Leu Glu Gln Val Gln Ser
 165 170 175
 Ile Glu Gln Ser Leu Asp Glu Ser Gly Leu Glu Ala Leu Ser Ser Phe
 180 185 190
 Leu Lys Thr Lys Lys Asn Leu Asp Gln Leu Glu Asp Ala Lys Asp Leu
 195 200 205
 Asp Glu Asn Gly Gly Val Gln Val Leu Leu Lys Ala Leu Leu Ser Leu
 210 215 220
 Ser Tyr Leu Arg Ser Ile Leu Met Lys Gly Leu Glu Ser Gly Leu Arg
 225 230 235 240
 Asn Asp Ala Pro Asp Ser Ala Ile Ala Met Arg Gln Lys Trp Arg Leu
 245 250 255
 Cys Glu Ile Gly Leu Glu Asp Tyr Ser Phe Val Leu Leu Ser Arg Tyr
 260 265 270
 Ile Asn Ala Leu Glu Ala Leu Gly Gly Ser Ala Ser Leu Ala Glu Gly
 275 280 285
 Leu Pro Thr Asn Thr Ser Leu Trp Asp Asp Ala Leu Asp Ala Leu Val
 290 295 300
 Ile Gly Ile Asn Gln Val Ser Phe Ser Gly Trp Lys Pro Asn Glu Cys
 305 310 315 320
 Thr Ala Ile Val Asn Glu Leu Leu Ser Trp Lys Gln Lys Gly Leu Ser
 325 330 335
 Glu Phe Glu Gly Ser Glu Asp Gly Lys Tyr Ile Trp Ala Leu Arg Leu
 340 345 350
 Lys Ala Thr Leu Asp Arg Ser Arg Arg Leu Thr Glu Glu Tyr Ser Glu
 355 360 365
 Ala Leu Leu Ser Ile Phe Pro Glu Lys Val Lys Val Leu Gly Lys Ala
 370 375 380

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

Leu Gly Ile Pro Glu Asn Ser Val Arg Thr Tyr Thr Glu Ala Glu Ile
 385 390 395 400

Arg Ala Gly Val Ile Phe His Val Ser Lys Leu Cys Thr Val Leu Leu
 405 410 415

Lys Ala Thr Arg Ala Val Leu Gly Ser Ser Val Trp Asp Val Leu Val
 420 425 430

Pro Gly Val Ala His Gly Ala Leu Ile Gln Val Glu Arg Ile Ala Pro
 435 440 445

Gly Ser Leu Pro Ser Ser Ile Lys Glu Pro Val Val Leu Val Val Asn
 450 455 460

Lys Ala Asp Gly Asp Glu Glu Val Lys Ala Ala Gly Asp Asn Ile Val
 465 470 475 480

Gly Val Ile Leu Leu Gln Glu Leu Pro His Leu Ser His Leu Gly Val
 485 490 495

Arg Ala Arg Gln Glu Lys Val Val Phe Val Thr Cys
 500 505

<210> 23

<211> 8

<212> PRT

<213> Triticum aestivum

<400> 23

Arg Asn Asp Ala Thr Asp Ala Gly
 1 5

<210> 24

<211> 8

<212> PRT

<213> Triticum aestivum

<400> 24

Gly Asn Thr Ser Val Trp Asp Asp
 1 5

<210> 25

<211> 509

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

<212> DNA

<213> Triticum aestivum

<220>

<221> CDS

<222> (1)..(507)

<223>

<400> 25

aat ggc gct ttt gtc gaa caa ttt caa ata ttt tat agc gaa cta aaa	48
Asn Gly Ala Phe Val Glu Gln Phe Gln Ile Phe Tyr Ser Glu Leu Lys	
1 5 10 15	

gac ttc ttt aat gcc ggc agc ctg ttt gaa caa ctg gaa tcc atc aag	96
Asp Phe Phe Asn Ala Gly Ser Leu Phe Glu Gln Leu Glu Ser Ile Lys	
20 25 30	

gaa tct ttg aat gat tct ggc tta gaa gca ctg tca tca ttt gtc aaa	144
Glu Ser Leu Asn Asp Ser Gly Leu Glu Ala Leu Ser Ser Phe Val Lys	
35 40 45	

acc aaa cag agt ttg gac caa gtg gat gct gcg aac att caa gtt gtg	192
Thr Lys Gln Ser Leu Asp Gln Val Asp Ala Ala Asn Ile Gln Val Val	
50 55 60	

atg aag acc ttg cag tca ttg tct tca ttg aga tca gtt cta atg aag	240
Met Lys Thr Leu Gln Ser Leu Ser Ser Leu Arg Ser Val Leu Met Lys	
65 70 75 80	

ggc ctt gaa agt ggc ctt aga aat gat gcg act gat gcc ggt ata gca	288
Gly Leu Glu Ser Gly Leu Arg Asn Asp Ala Thr Asp Ala Gly Ile Ala	
85 90 95	

atg cga caa aag tgg cgc ctt tgt gag att ggt ctt gag gat tat tct	336
Met Arg Gln Lys Trp Arg Leu Cys Glu Ile Gly Leu Glu Asp Tyr Ser	
100 105 110	

ttt gtt ttg tta agc aga tat atc aat ggt ctt gaa gct tca ggt gga	384
Phe Val Leu Leu Ser Arg Tyr Ile Asn Gly Leu Glu Ala Ser Gly Gly	
115 120 125	

tca gct tca ctt gca caa tgt gtg gct gga aat aca agt gta tgg gac	432
Ser Ala Ser Leu Ala Gln Cys Val Ala Gly Asn Thr Ser Val Trp Asp	
130 135 140	

gat acc ctt gat gcc ctt att att ggc gtc aat caa gtt agc ttt tca	480
Asp Thr Leu Asp Ala Leu Ile Ile Gly Val Asn Gln Val Ser Phe Ser	
145 150 155 160	

ggt tgg aag cca gag gaa tgc att gct at	509
Gly Trp Lys Pro Glu Glu Cys Ile Ala	
165	

<210> 26

<211> 169

<212> PRT

BCS 04-501-PCT_SEQUENZPROTOKOLL_Verfahren zur Identifizierung.ST25

<213> Triticum aestivum

<400> 26

Asn Gly Ala Phe Val Glu Gln Phe Gln Ile Phe Tyr Ser Glu Leu Lys
 1 5 10 15
 Asp Phe Phe Asn Ala Gly Ser Leu Phe Glu Gln Leu Glu Ser Ile Lys
 20 25 30
 Glu Ser Leu Asn Asp Ser Gly Leu Glu Ala Leu Ser Ser Phe Val Lys
 35 40 45
 Thr Lys Gln Ser Leu Asp Gln Val Asp Ala Ala Asn Ile Gln Val Val
 50 55 60
 Met Lys Thr Leu Gln Ser Leu Ser Ser Leu Arg Ser Val Leu Met Lys
 65 70 75 80
 Gly Leu Glu Ser Gly Leu Arg Asn Asp Ala Thr Asp Ala Gly Ile Ala
 85 90 95
 Met Arg Gln Lys Trp Arg Leu Cys Glu Ile Gly Leu Glu Asp Tyr Ser
 100 105 110
 Phe Val Leu Leu Ser Arg Tyr Ile Asn Gly Leu Glu Ala Ser Gly Gly
 115 120 125
 Ser Ala Ser Leu Ala Gln Cys Val Ala Gly Asn Thr Ser Val Trp Asp
 130 135 140
 Asp Thr Leu Asp Ala Leu Ile Ile Gly Val Asn Gln Val Ser Phe Ser
 145 150 155 160
 Gly Trp Lys Pro Glu Glu Cys Ile Ala
 165

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

PCT/EP2005/002454



INTERNATIONAL FORM

BAYER Cropscience GmbH
Brüningstr. 50

65929 Frankfurt/Main

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: BAYER Cropscience GmbH Brüningstr. 50 Address: 65929 Frankfurt/Main	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 16264 Date of the deposit or the transfer ¹ : 2004-03-03
III. VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 2004-03-04 ¹ . On that date, the said microorganism was <input checked="" type="checkbox"/> viable <input type="checkbox"/> no longer viable	
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED ⁴	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2004-03-08

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.

³ Mark with a cross the applicable box.

⁴ Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE**DSMZ**Deutsche Sammlung von
Mikroorganismen und
Zellkulturen GmbH

INTERNATIONAL FORM

BAYER Cropscience GmbH

Brüningstr. 50

65929 Frankfurt/Main

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: A.t.-ok1 pGEM	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 16264
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: (<input checked="" type="checkbox"/>) a scientific description () a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2004-03-03 (Date of the original deposit) ¹ .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2004-03-08

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.



INTERNATIONAL FORM

Bayer Cropscience GmbH

Brüningstr. 50

65929 Frankfurt/Main

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: pMI50	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 16302
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I. above was accompanied by: (<input checked="" type="checkbox"/>) a scientific description (<input type="checkbox"/>) a proposed taxonomic designation (Mark with a cross where applicable).	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2004-03-19 (Date of the original deposit).	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____ (date of receipt of request for conversion).	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 2004-03-24

¹ Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

INTERNATIONAL FORM

Bayer Cropscience GmbH
Brünigstr. 50

65929 Frankfurt/Main

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR		II. IDENTIFICATION OF THE MICROORGANISM	
Name: Bayer Cropscience GmbH Address: Brünigstr. 50 65929 Frankfurt/Main		Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 16302 Date of the deposit or the transfer: 2004-03-19	
III. VIABILITY STATEMENT			
The viability of the microorganism identified under II above was tested on 2004-03-23 On that date, the said microorganism was (X) viable () no longer viable			
IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED¹			
V. INTERNATIONAL DEPOSITARY AUTHORITY			
Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig		Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wechs Date: 2004-03-24	

¹ Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
² In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
³ Mark with a cross the applicable box.
⁴ Fill in if the information has been requested and if the results of the test were negative.

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